UNRAVELING THE MECHANISM OF IL-12 MEDIATED GLIOMA REJECTION

Dissertation

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1. Summary

Glioblastoma is a highly destructive primary brain cancer with poor prognosis despite aggressive treatment consisting of surgical resection, radiation, and chemotherapy. Hence, development of novel treatment strategies represents a continuous need. One treatment approach aims to revert the suppressive tumor microenvironment into a pro-inflammatory microenvironment leading to tumor rejection. Multiple clinical trials are currently ongoing using intratumoral delivery of the pro-inflammatory cytokine interleukin (IL)-12 with the aim of minimizing toxicity while maximizing efficacy. However, improvement of IL-12 based immunotherapy requires an immunological understanding of the mechanistic underpinnings. Using a syngeneic mouse model for glioblastoma, we previously showed that intratumoral delivery of IL-12 leads to tumor rejection. Here we show that tumor control is dependent on IL-12 signaling in tumor-invading (cluster of differentiation 8) $CD8^+$ T cells, driving local expansion. We observed a concomitant influx of myeloid cells into the tumor microenvironment. However, chemokine-receptor 2 (CCR2)-dependent monocyte-derived cells were dispensable for IL-12-mediated tumor rejection. Tumor control was supported by $CD103^+$ dendritic cells (DCs) found within the tumor microenvironment. However, in the absence of $CD103^+$ DCs mice seem to control, but are not able to reject IL-12 expressing tumors.

Our findings provide new insights into the mechanism of IL-12 mediated glioma rejection, including requirement of directly and indirectly responsive cell types, to consider for the development of novel treatment strategies.

2. Zusammenfassung

Das Glioblastom ist ein bösartiger Hirntumor, der trotz aggressiver Behandlung bestehend aus chirurgischer Resektion, Strahlentherapie und Chemotherapie schlechte Prognosen aufzeigt. Daher ist es essenziell kontinuierlich neue Behandlungsstrategien zu entwickeln. Ein Behandlungsansatz zielt darauf ab die suppressive Tumormikroumgebung in eine proinflammatorische Mikroumgebung umzuwandeln, die zur Abstossung des Tumors führt. Aktuelle klinische Studien basieren auf der intratumoralen Verabreichung des pro-inflammatorischen Zytokin Interleukin (IL) -12. Das Ziel ist, die Toxizität zu minimieren und gleichzeitig die therapeutische Wirksamkeit zu maximieren. Voraussetzung für die Entwicklung der IL-12-basierten Immuntherapie ist das Verständnis der grundlegenden immunologischen Mechanismen. Mit einem Mausmodell für das Glioblastom konnten wir nachweisen, dass die intratumorale Verabreichung von IL-12 zur Tumorabstossung führt.

In der vorliegenden Studie zeigen wir, dass die IL-12 induzierte Tumorkontrolle von der Wirkung auf tumor-infiltrierende $CD8^+$ T-Zellen basiert und zu deren Expansion führt. Gleichzeitig war eine Anhäufung von myeloiden Zellen in der Tumormikroumgebung zu beobachten, wobei CCR2-abbhängige monozytische Zellen für die IL-12 vermittelte Tumorabstossung entbehrlich waren. Die Tumorkontrolle wird von $CD103^+$ dendritischen Zellen innerhalb der Tumormikroumgebung unterstützt, wobei in Abwesenheit von $CD103^+$ dendritischen Zellen eine Tumorkontrolle, aber keine Abstossung von IL-12 überexprimierenden Tumoren stattfindet.

Unsere Ergebnisse liefern neue Erkenntnisse über den Mechanismus der IL-12-vermittelten Gliomabstossung, einschliesslich der direkt und indirekt involvierten Zelltypen, die für die Entwicklung neuer Therapiestrategien Berücksichtigtung finden sollten.

3. Abbreviations

APC	Antigen presenting cell
ATP	adenosine 5´-triphosphate
αGalCer	α-galactosylceramide
Batf3	Basic Leucine Zipper Factor ATF-Like Transcription 3
BBB	Blood brain barrier
BLI	Bioluminescence imaging
BM	Bone marrow
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CCL2	Chemokine (C-C) motif ligand 2
CCR2	Chemokine (C-C) motif receptor 2
cDC	Classical dendritic cell
CNS	Central nervous system
cLN	Cervical lymph node
CD	Cluster of differentiation
CT	Computed Tomography
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAMPs	Damage-associated molecular
DC	Dendritic cell
EBI3	Epstein-Barr Virus Induced 3
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent Assay
FCS	Fetal calf serum
Flt3l	FMS-like tyrosine kinase 3 ligand
FoxP3	Forkhead box P3
FTY720	Fingolimod
GFAP	Glial fibrillary acidic protein
GL-261	Glioma-261
GM-CSF	Granulocyte/macrophage colony stimulating factor
gp100	Glycoprotein 100
HMGB1	High mobility group box 1 protein
HSV	Herpes simplex virus
i.c.	Intracranial
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cell
IFN	Interferon
IP-10	Interferon gamma-induced protein
STAT	Signal transducer and activator of
i.v.	intravenous
IPP	Isopentenyl
KIR	Killer-cell like receptors
IRF8	Interferon regulatory Factor 8
LN	Lymph node
MHC	Major histocompatibility complex
moDC	Monocyte-derived
MAP	Mitogen-activated protein
MCA	3-methylcholanthrene
MDSC	Myeloid-derived cell
MICA	MHC class I polypeptide-related sequence A
MIP-1α	Macrophage proteins
MRI	Magnetic resonance imaging
NKG2D	Natural-killer group 2, member D
NK cell	Natural killer cell
NKT cell	Natural killer T cell
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PD-1	Programmed cell death
pDC	Plasmacytoid dendritic
PD-L1	Programmed death ligand
PET	Positron-emission tomography
rAAV	Recombinant adeno-associated virus
Rag	recombination-activating gene
RAE-1	Retinoic acid early inducible 1
RB	Retinoblastoma-
ROI	Region of interest
RT	Room temperature
SIRPα	Signal regulatory protein
s.c.	subcutaneous
SFV	Semliki Forest virus

	transcription
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIL	Tumor-infiltrating lymphocyte
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP53	Tumor suppressor 53
Tregs	Regulatory T cells
TRP2	Tyrosinase-related protein 2
VEGF	Vascular endothelial growth factor
VSV	vesicular stomatitis virus
WT	Wild type
Zbtb46	Zinc finger and BTB domain containing 46

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5. Introduction

5.1. Overview of cancer

To date, cancer remains the second leading cause of death worldwide, responsible for 8.8 million deaths in 2015. Arising from a single abnormal cell dividing without control, the primary risk factor for developing cancer is increasing age (86% of cancers diagnosed in the U.S. affect people older than 50 years). However, external factors, such as tobacco or physical inactivity and internal factors, such as genetic predisposition or immune conditions, can increase the risk of cancer. Based on the overall experience in the general population, the lifetime risk of developing cancer has been estimated to be 42% in men and 38% in women in the U.S. [1].

In the past decades, overall patient survival has significantly improved across cancer types. In this respect, the 5-year relative survival rate combining all cancers increased from 49% during 1975-1977 to 69% during 2005-2011 [1]. Improved survival rates are mainly due to advances in technology, leading to early detection and prevention [2]. Moreover, advances in understanding the genetics of cancer development, the possibility of expression profiling of cancer cells and increased understanding regarding the involvement of the immune system during cancer progression, have paved the way for targeted therapies and immunotherapies in addition to surgery, radiation, and chemotherapy [2]. However, many cancers, among them highly malignant tumors of the CNS remain incurable despite aggressive therapy.

5.1.1. The hallmarks of cancer

Cancer development seen from the evolutionary perspective comprises the stepwise acquisition of biological capabilities. The transition from a normal to a neoplastic cell is believed to require traits including sustained proliferative signaling, replicative immortality, resisting cell death, inducing angiogenesis, evasion of growth suppressors and activation of invasion and metastasis [3]. Summarized in the hallmarks of cancer, the acquisition of these six traits were recently supplemented by the hallmarks of reprogramming of energy metabolism and evasion of immune destruction, as well as the enabling characteristics of genome instability and tumor-promoting inflammation (Figure 1) [4].

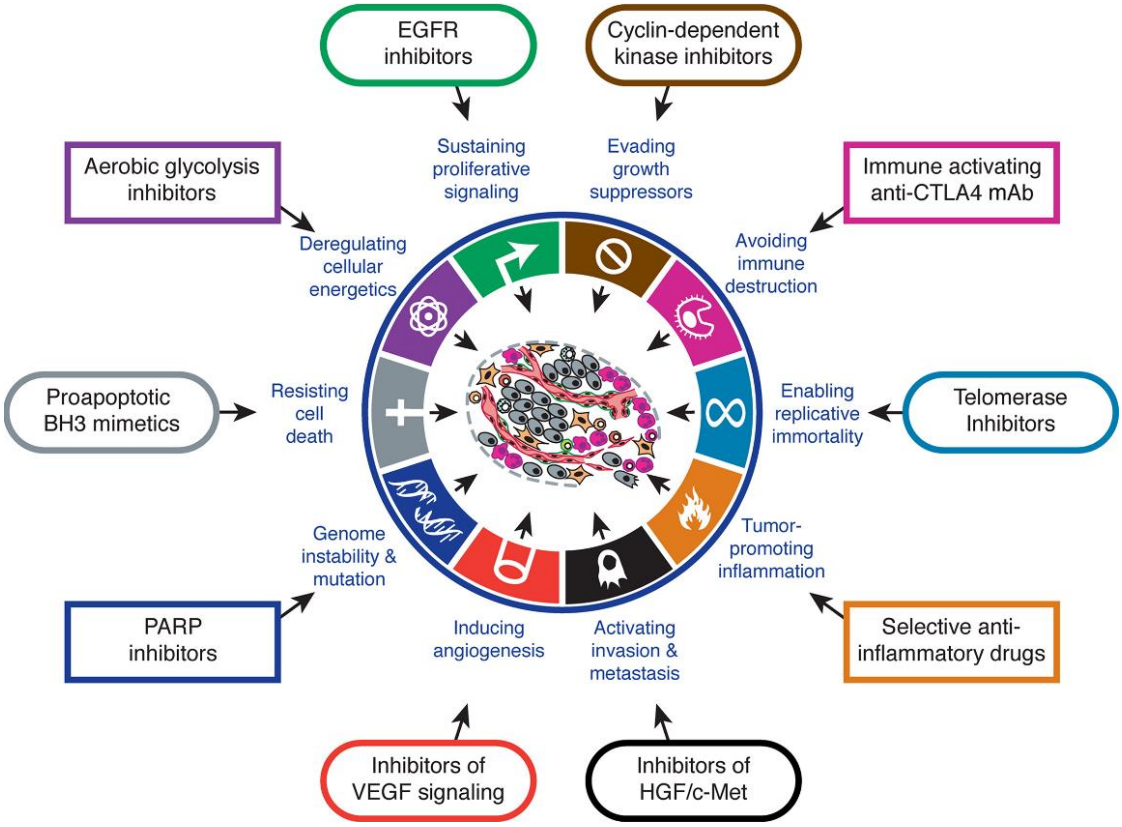

Figure 1**: The hallmarks of cancer.** Initially described as the six hallmarks, consisting of sustained proliferative signaling, replicative immortality, evasion of growth suppressors, resisting cell death, inducing angiogenesis and activation of invasion and metastasis in 2000 by Hanahan and Weinberg [3]. Emerging concepts were added in 2011 and included genome instability and mutation, tumor-promoting inflammation, reprogramming energy metabolism and evading immune destruction [4]. Adapted from [4].

5.1.1.1. Sustaining proliferative signaling

Sustained proliferative signaling represents the most prominent acquired trait of cancer cells. While healthy tissues possess multiple regulatory mechanisms to control cell numbers, cancer cells exploit numerous ways to ensure abnormal proliferation. For example, defects in negative-feedback mechanisms account for the uncontrolled proliferation of cancer cells. Moreover, cancer cells may regulate their growth by releasing growth factor ligands and simultaneously expressing the cognate receptor, thus inducing autocrine proliferation [4, 5]. Overexpression of the receptor itself may enable cancer cells to become hyper-responsive to growth factors [6]. Alternatively, cancer cells may stimulate the production of growth factors by surrounding healthy tissue [4, 7, 8].

The most studied regulators of the proliferative signaling circuitry are the GTPases H-Ras, K-Ras, and N-Ras, with *K-Ras* mutations being most abundant in several types of cancer [9]. Constitutive activation due to mutations at codons 12, 13 or 61 has multiple

effects such as activation of Raf kinases leading to activation of the mitogen-activated protein (MAP)-kinase pathway resulting in increased proliferation. Additionally, activation of the MAP-kinase pathway can be a result of somatic mutations in the *BRAF* gene itself. Notably, constitutive activation of the serine/threonine kinase BRAF (V600E) is found in 50% of epithelioid glioblastomas and 66% of malignant melanomas (V600E) [10-13].

5.1.1.2. Evading growth suppressors

Apart from ensuring sustained proliferative signaling, cancer cells also need to acquire the capacity to evade growth suppressors. Evasion frequently involves the tumor suppressor retinoblastoma-associated (RB) and TP53 proteins. While RB is responsible for the decision of cell cycle progression (or cell cycle arrest) influenced primarily by extracellular-derived inhibitory signals, TP53 mainly acts upon intracellular signals, e.g., related to stress. However, apart from decisions on cell cycle progression or arrest (e.g., due to extensive genome damage), TP53 may also lead the cell into programmed cell death in case of irreparable damage (further described in 5.1.1.3) [4]. Notably, *TP53* mutations leading to loss-of-function represents an early and frequent genetic alteration occurring in 60% of low-grade astrocytomas [14]. An additional mechanism leading to evasion of growth suppressors results from mutations in genes such as *NF2*. In healthy cells, NF2 is part of the contact inhibition mechanism leading to cell cycle arrest when cells reach a high density. In cancer cells, loss-of-function of NF2, and thereby loss of contact inhibition, leads to uncontrolled growth [4].

5.1.1.3. Resisting cell death

Programmed cell death (apoptosis) defines the orderly and efficient removal of damaged cells [4, 15]. Triggered by extracellular factors (such as Fas ligand/Fas receptor interaction) or intracellular signals (e.g., genotoxic stress), the apoptotic machinery requires a controlled counterbalance of proteins responsible for the inhibition (e.g., Bcl-2) or induction (such as Bax and Bak) of apoptosis [4, 16]. Deregulation of apoptosis, and thereby resistance of cancer cells to cell death has been established as a hallmark of cancer, responsible for tumor development, progression and resistance to therapy [15]. In this respect, mutations of *Bcl-2* have been identified to confer resistance of melanoma cells to the chemotherapeutic paclitaxel [17].

5.1.1.4. Enabling replicative immortality

Healthy cells may only run through a limited number of growth-and-division cycles before entering a constant non-proliferative state (senescence) or a crisis phase leading to cell death [4]. Responsible for the limited proliferation capacity are telomeres, a region of repetitive nucleotide sequences that protect the end of chromosomes. Every division shortens telomere length until telomeres cannot protect chromosome ends anymore, leading the cell to senescence or crisis. Cancer cells circumvent this limited proliferative capacity by expressing significant levels of the enzyme telomerase, which adds telomere repeats, preventing senescence or crisis [3, 4]. Notably, mutations in the promoter of the telomerase reverse transcriptase gene leading to increased expression of telomerase account for a frequent event in many tumor types, e.g., found in 83% of glioblastoma samples [18, 19].

5.1.1.5. Inducing angiogenesis

Normal tissues and tumors require a constant supply of nutrients and oxygen for their growth and survival. In healthy tissues, the development of vasculature is a tightly regulated process, occurring during embryogenesis and only transiently in adults, e.g., during wound healing. The formation of vasculature encompasses the steps of new endothelial cells arising, assembly of endothelial cells into tubes (vasculogenesis) and the growth of new vessels from existing ones (angiogenesis). To sustain the supply of nutrients and oxygen for neoplastic growth, tumors frequently activate the “angiogenic switch” driving the formation of new vessels [3, 4]. One of the most prominent angiogenic regulators is the vascular endothelial growth factor-A (VEGF-A). In this regard, angiogenesis in human glioma involves a paracrine mechanism of VEGF produced by glioma cells and flt-1, a tyrosine-kinase receptor for VEGF expressed in endothelial cells [20]. Inhibitors targeting VEGF signaling (Bevacizumab) are approved by the FDA as a treatment for glioblastoma in some countries. However, development of resistance to such therapy suggests that tumors can adapt to bypass antiangiogenic signals [21].

5.1.1.6. Activating invasion and metastasis

Metastasis, the formation of secondary tumors in distinct organs, is tightly connected to mortality and morbidity in cancer patients [22]. While two decades ago, the underlying mechanisms were mostly unclear, we now know that invasion and metastasis occurs in a

sequential cascade. Beginning with the invasion of cancer cells into the surrounding tissues, cancer cells intravasate into the blood and lymphatic vessels. Then, cancer cells transit through the lymphatic and hematogenous system, before extravasating into the parenchyma of distant tissues forming micrometastasis and eventually macroscopic tumors [4]. One of the best-characterized alterations found in transformed cells associated with invasion and metastasis describes the cell-to-cell adhesion molecule E-cadherin, responsible for binding cells within tissues. In this regard, downregulation or mutational inactivation of E-cadherin is found in multiple aggressive human carcinomas [4, 23].
Regarding the invasion and metastasis into the CNS, secondary tumors are found in ~15% of cancer patients. Among solid tumors, lung and breast cancers are the most common to metastasize to the CNS [24].

5.1.1.7. Deregulating cellular energetics

In addition to providing sufficient nutrients and oxygen through increased angiogenesis (section 5.1.1.5), neoplastic cells are required to adjust their energy metabolism to ensure cell growth and division. Under normal conditions, cells metabolize glucose via glycolysis to pyruvate and subsequently to carbon dioxide in the process of oxidative phosphorylation. Depending on the availability of oxygen, this reaction yields high amounts of adenosine 5´-triphosphate (ATP), the cell's energy source. In cancer cells, however, a metabolic switch occurs even in the presence of oxygen. By reprogramming their metabolism, cancer cells reach a state of aerobic glycolysis, also referred to as the Warburg effect [25]. In this respect, cancer cells upregulate glycolysis and lactic acid fermentation instead of normal aerobic respiration, ultimately yielding lower amounts of ATP. To compensate lower ATP levels, cancer cells frequently upregulate glucose transporters leading to increased amounts of glucose in the cytoplasm. This knowledge has led to the development of widely used diagnostic tools like positron emission tomography (PET) with a radiolabeled glucose analog to detect increased uptake and metabolism of glucose in many human tumors [4]. Notably, upregulation of the glucose transporter GLUT3 has been correlated with poor survival in brain tumors [26]. One explanation for this metabolic switch is the frequently occurring hypoxia in solid tumors. Alternatively, increased availability of products related to glycolysis might support the synthesis of macromolecules and organelles necessary for assembling new cells [4].

5.1.1.8. Genome instability and mutation

The successful development of neoplastic cells relies on either mutational or epigenetic change affecting the regulation of gene expression. In addition to loss-of-function of tumor suppressors like TP53 (described in section 5.1.1.2), frequent inactivating mutations or epigenetic repressions occur in the genome maintenance systems, the so-called “caretakers” of the genome [4]. Defects in caretaker genes can, for instance, influence the capability of detecting DNA damage and prevent the repair of damaged DNA [4, 27]. Conversely, introducing mutant copies of caretaker genes into mouse germline has been shown to increase cancer incidence [4, 28], underlining the importance of genome instability as enabling characteristic of carcinogenesis.

5.1.1.9. Tumor-promoting inflammation

Neoplastic lesions can contain varying numbers of immune cells ranging from subtle infiltration to gross inflammation. While the infiltration of immune cells was interpreted as an attempt of the immune system to attack a neoplastic lesion, it is now believed that tumor-associated inflammation can also enhance tumorigenesis and progression. In this respect, inflammation can support the hallmarks described above in multiple ways, such as through the supply of growth factors supporting sustained proliferative signaling or by providing survival factors preventing cell death [4]. A detailed description of tumor-promoting inflammation will follow in section 5.2.1.3.

5.1.1.10. Evading immune destruction

Given the attempts of the immune system to eradicate tumors, tumors are believed to be under constant immune selection pressure to evade detection. How components of the immune system are disabled, preventing tumor eradication, will be described in detail in section 5.2.1.3.

In summary, the hallmarks of cancer describe the acquisition of biological capabilities that lead to the development of a clinically apparent malignancy. Given the complexity of tumor-host immunological interactions, the involvement of the immune system during cancer progression will be described in the following sections.

5.2. The immune system and cancer

5.2.1. From cancer immunosurveillance to immunoediting

Already in the early 1900s, it has been suggested by Paul Ehrlich that the immune system plays a crucial role in controlling cancer, by postulating that cancer would be frequent in long-lived organisms without protective immunity [29]. However, since function and components of the immune system remained mostly elusive, it was not possible to verify this hypothesis. Improved understanding of the components of the immune system and availability of inbred mouse strains led MacFarlane Burnet and Lewis Thomas to the theory of "cancer immunosurveillance," postulating that adaptive immunity is preventing cancer development [30, 31]. Crucial for the tenet of tumor immunology was the discovery of tumor antigens, proven by injecting mice with chemically induced tumors. In these experiments, subsequent re-challenge with the same tumor led to tumor protection, thus proving the existence of tumor antigens [32, 33]. Nevertheless, the concept of cancer immunosurveillance received great skepticism, arguing that cancer susceptibility of spontaneous and carcinogen-induced tumors was similar in immunocompetent and immunodeficient mice [33-35]. Moreover, it was reasoned that tumor cells do not possess the required danger signals to activate the immune system [36]. Others argued that tumor cells were resembling healthy cells to the extent that tumor cells could not be recognized as foreign [37]. Additionally, the observation of persistent activation of innate immunity promoting cellular transformation and tumor outgrowth was explained by precluding a protective function of the immune system [33, 38, 39].

In the 1990s, however, the concept of cancer immunosurveillance was reconsidered. Due to advances in transgenic mouse technology resulting in the availability of mouse models for distinct immunodeficiencies on pure genetic backgrounds and the use of monoclonal antibodies, cytokines, and cell types were now associated with a protective role of the immune system against tumor formation. It was shown that mice lacking adaptive immunity and mice deficient in the pro-inflammatory cytokine interferon (IFN)-γ were more susceptible to carcinogen-induced and spontaneous tumors [33, 40, 41].

In 2001, the role of the immune system in cancer was reassessed once again, based on the discovery that the immune system is capable of suppressing cancer growth and selecting tumor cells more fit to survive [33, 41]. In the underlying experiments, both immunocompetent and immunodeficient mice were subjected to a primary carcinogen-induced tumor challenge. Tumor cell lines were established from both groups and

subsequently injected into naïve WT recipients. Monitoring of the growth profile led to the finding that tumor cells derived from immunodeficient mice were rejected by naïve WT recipients, while mice receiving tumor cells initially derived from immunocompetent mice were not able to reject the tumor. It was concluded that tumor cells from mice with a compromised immune system had more immunogenic, "unedited" profile, compared to mice with an intact immune system bearing "edited" tumors. These experiments provided the foundation of the cancer immunoediting hypothesis, consisting of the 3 phased "elimination, "equilibrium" and "escape," shown in Figure 2 [33, 41, 42].

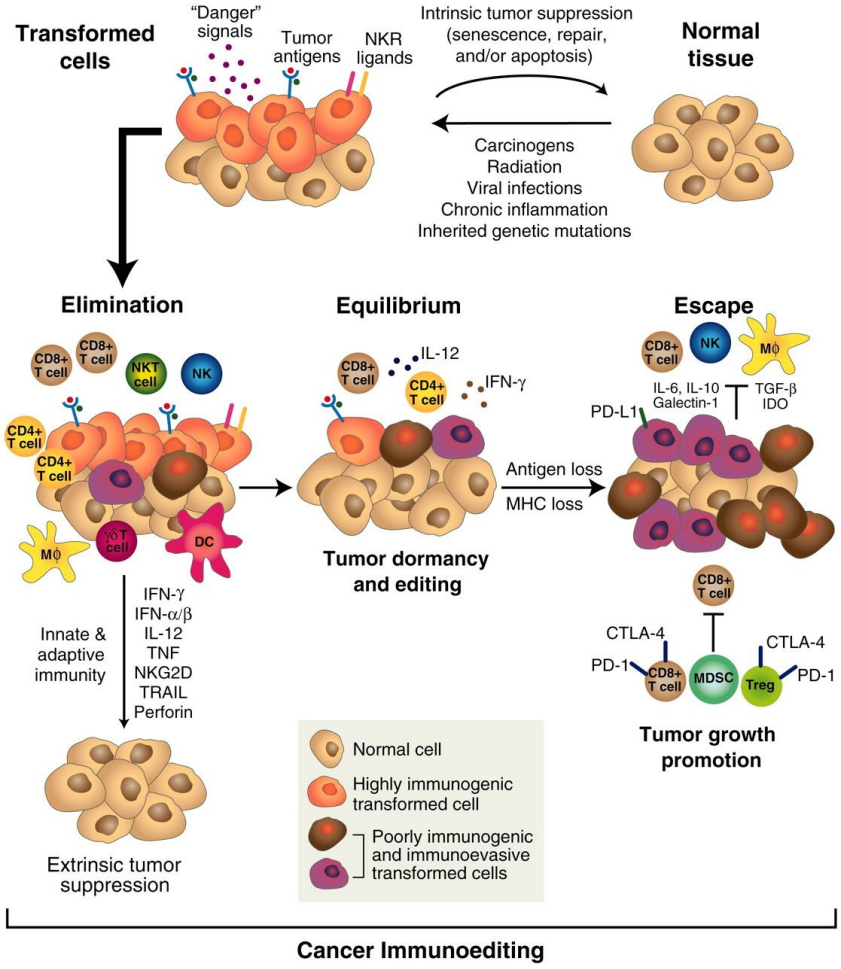

Figure 2: **The concept of cancer immunoediting.** Carcinogen or radiation-induced challenge can lead to the development of transformed cells given that mechanisms of intrinsic tumor suppression fail. These highly immunogenic transformed cells can release danger signals, express tumor-specific antigens and ligands activating the innate arm of immunity. Within this so-called elimination phase of cancer immunoediting, both innate and adaptive immunity participate in extrinsic tumor suppression mechanisms, e.g., through the release of perforin into the target cell inducing its apoptosis. Rare variants not eliminated can enter the equilibrium phase. During this phase, tumor outgrowth is prevented by the adaptive arm of immunity. Notably, editing of tumor immunogenicity merely occurs in the equilibrium phase. Concomitant immune selection pressure and genetic instability of tumor cells allow rare variants to enter the escape phase, in which the tumor uses an intrinsic mechanism like antigen loss, but also tumor-promoting inflammation recruiting immuno-suppressive cells to form a clinical malignancy. Adapted from [33].

5.2.1.1. Elimination

Elimination represents the first phase of cancer immunoediting. Given that intrinsic tumor suppression mechanisms fail to maintain normal somatic tissue, this phase is characterized by localizing, recognizing and destroying transformed cells to prevent the development of malignancy. Even though the underlying mechanisms of how early tumor development is being prevented are not fully understood, many of the molecules participating in the elimination phase have been discovered. These discoveries derived for instance from mouse models lacking immune cell subsets, recognition molecules, effector pathways or cytokines, which were compared to wild-type mice in regards to tumor initiation, growth, and metastases [33, 43]. Apart from the dependency of elimination on tumor characteristics such as anatomic location and growth rate, these experiments showed that the elimination phase involves both, innate and adaptive immunity. In this respect, localization of transformed cells has been associated with classical danger signals such as Type 1 IFNs, found early during tumor development. Type 1 IFNs activate dendritic cells (DCs) and thus induce adaptive anti-tumor immune responses [36]. Also, damage-associated molecular pattern molecules (DAMPs) released by dying tumor cells, e.g., the high mobility group box 1(HMGB1) have been associated with the elimination phase. DAMPs alert the immune system to the presence of dying tumors cells, triggering immunogenic cell death [44].

Importantly, the elimination phase is characterized by the infiltration of multiple immune cell subsets. Triggered by tissue disruption of invasively growing tumor cells, inflammatory signals released recruit cells of the innate immune system to the tumor site. Natural killer (NK) cells, NKT cells, gamma-delta (γδ) T cells, macrophages and dendritic cells (DCs) are among these first infiltrates, subsequently recruiting cells of adaptive immunity recognizing specific targets and eliminating transformed cells. The main characteristics of these infiltrating cells, as well as their contribution to extrinsic tumor suppression, will be described below.

Natural killer cells

NK cells are lymphocytes belonging to the innate immune system. Involved in surveying the body for stressed and abnormal cells, NK cells integrate signals from various activating and inhibitory receptors. Primary activating receptors include for instance NKG2D, binding to stress ligands such as retinoid acid early-inducible protein (RAE)-1

(mouse) and MHC-class-I-polypeptide-related sequence A (MICA, human), frequently expressed on tumor cells due to constitutive activation of DNA-damage response pathways [45]. Activating receptor signaling in NK cells can induce apoptosis in target cells through the release of the lytic granules containing perforin and granzymes. Perforin release induces apoptosis by creating pores in the plasma membrane, while granzymes enter the target cells triggering apoptotic pathways by cleaving precursors of caspases. Another NK cell-related effector mechanism is the secretion of various cytokines and chemokines such as IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage inflammatory protein (MIP)-1α [46]. Inhibitory receptors such as members of the Killer Cell Immunoglobulin-like-Receptors (KIR) family (human) and the C-type lectin-like Ly49 (mouse) bind to Major Histocompatibility Complex class I molecules (MHCI) expressed on healthy cells, preventing NK cell activation. However, both transformed and virally infected cells frequently display reduced or altered expression of MHCI, leading to the reduced presentation of self-peptides. NK cells detect changes in self-molecules, attacking virus-infected and transformed cells with impaired MHCI expression, as described in the "missing-self hypothesis" [47].

In preclinical models, antibody-mediated depletion of NK cells has been shown to result in the increased susceptibility to MCS-induced sarcomas. Whereas RAE-1 transgenic mice, characterized by the defective killing of the NKG2D pathway, display increased DMBA/TPA (7,12-di-methylbenza-anthracene/12-O-tetradecanoyl-phorbol-13-acetate) -induced skin tumors [48].

Natural killer T cells

NKT cells share both features of NK and T cells and are thus grouped into the family of innate-like lymphocytes. NKT cells express both NK1.1, a classical NK cell marker (murine C57BL/6) and the αβ T cell receptor (TCR). Expressing the semi-invariant TCRα chain (Vα14-Jα18 TCR in mice, Vα24-Jα18 in humans) together with the Vβ chains (Vβ8,7 and 2 in mice; Vβ11 in humans), NKT cells preferentially recognize glycolipids presented by the non-classical MHC-like molecule CD1d, such as α-galactosylceramide (α-GalCer) [49]. NKT cells activate cytotoxic functions like NK cells in a perforin-dependent fashion. Additionally, NKT cells produce various cytokines, such as IFN-γ, TNF, Interleukins (IL)-2, -4, -10, -13, -17, -21, -22 and GM-CSF, thus secreting both Th1/pro-inflammatory and Th2/anti-inflammatory cytokines [49]. Moreover, NKT cells

can induce DC maturation, enhancing priming and $CD8^+$ T cell responses. In tumor immunity, type I NKT subsets promote tumor control, in contrast, to type II NKT cells which can suppress anti-tumor immune responses. Moreover, mice lacking invariant NKT cells (*Jα18*$^{-/-}$ mice) have an increased susceptibility to 3´-methylcholanthrene (MCA)-induced sarcomas compared to wild-type mice [50], underlining the role of invariant NKT cells during the elimination phase in some murine cancer models.

Gamma delta T cells

Gamma delta (γδ) T cells belong to the family of non-conventional or innate lymphocytes. Unlike conventional αβ T cells, γδ T cells do not express CD4 and CD8 lineage markers and rearrange the γ and the δ-chain segments of the TCR gene locus. However, like other T cell populations, activation and acquisition of effector functions depend on TCR engagement. Recognizing a variety of self and non-self-antigens, such as small peptides and phospholipids, γδ T cells recognize molecules frequently increased in tumors. For instance, metabolites of the isoprenoid pathway, such as isopentenyl pyrophosphate (IPP) can be sensed as a danger signal by γδ T cells [51]. Like NK cells, γδ T cells can also recognize stress ligands, such as RAE-1, through binding to the activating receptor NKG2D. Additionally, γδ T cells can kill infected, activated or transformed cells by triggering death-inducing receptors, such as Fas and TRAIL, and through the release of the cytotoxic molecules perforin and granzyme [52]. Lack of γδ T cells (*Tcrd*$^{-/-}$) has been associated with increased susceptibility to MCA-induced sarcomas and DMBA/TPA-induced skin tumors [53].

Macrophages

Macrophages are a crucial component of the innate immune system and prominent cells within tumors. Providing an immediate defense against pathogens, macrophages engulf and digest cellular debris, microbes, and cancerous cells. Macrophages recognize pathogen-associated molecular patterns (PAMPs) and detect endogenous danger signals present in necrotic tumor cells. In the tumor microenvironment, cellular composition and disease stage dictate whether macrophages adopt a tumor-suppressing (M1) or tumor-promoting phenotype (M2). M1 macrophages require IFN-γ for their development and secrete multiple pro-inflammatory cytokines, such as IL-12, IL-1β, and TNF-α, thus supporting the generation of a Th1 immune response [54]. Moreover, M1 macrophages

contribute to tumoricidal activity by producing reactive oxygen species [55]. In contrast, in the escape phase of cancer immunoediting, macrophages are biased towards an M2-polarized phenotype, promoting tumor growth and metastasis (described in section 5.2.1.3).

Despite not being related to the elimination phase of cancer immunoediting, therapeutic use of macrophages has been suggested in the context of blocking the CD47 molecule on tumor cells. Expression of CD47 functions as a “don`t eat me” signal upon interaction with the signal regulatory protein (SIRP)α inhibitory receptor on macrophages and blockade of CD47 can lead to a macrophage-dependent elimination of tumor cells [56, 57].

Dendritic cells

Dendritic cells are a subset of innate cells required for the initiation of T cell responses. Thus, DCs have a pivotal role in pathogen uptake and antigen presentation, linking innate and adaptive immune responses, presenting immunogenic epitopes in the context of MHCI and MHCII. As depicted in Figure 3, DCs are a group of heterogeneous cells, under steady-state conditions divided into lymphoid tissue-resident DCs and migratory DCs, and blood-derived DCs in inflammatory settings. For simplification purpose, this thesis will refer to the major subsets of migratory, resident DCs and monocyte-derived DCs (moDCs).

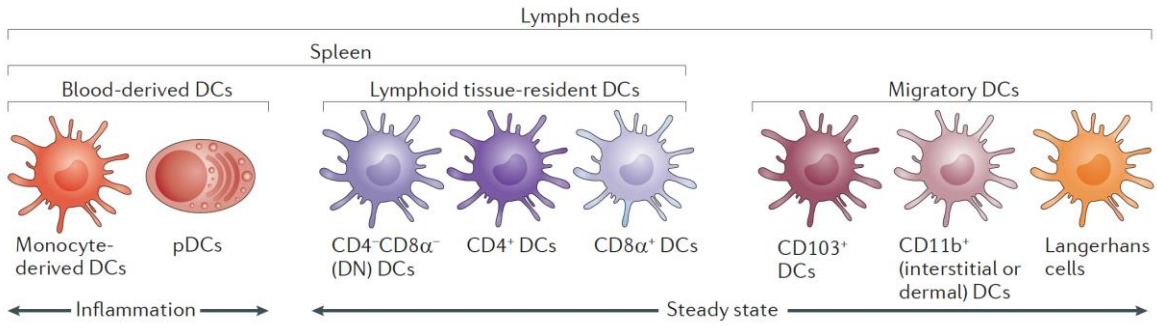

Figure 3: **Main subsets of mouse DCs.** Organization of DCs in lymph node and spleen under steady-state conditions and subsets associated with inflammation (adapted from [58]).

Migratory DCs sample antigens from peripheral tissues, migrating to draining lymph nodes via afferent lymphatics to induce T cell priming. Resident DCs are found in lymph nodes, spleen, and thymus. Depending on the subtype, resident DCs differ in the capacity to present antigens in the context of MHCI or MHCII.

MoDCs arise under inflammatory conditions and are blood-derived. Differentiating from monocytes in response to GM-CSF or Toll-like receptor 4 ligands, moDCs rapidly acquire prototypical features of DCs. Moreover, moDCs possess potent antigen-presenting capacity in the context of both MHCI and MHCII [58].

In the context of the elimination phase, DCs primarily function to induce an adaptive anti-tumor immune response. Responding to danger signals and cellular stress in the tumor microenvironment, DCs transport tumor antigens to draining lymph nodes for T cell activation. Moreover, it has been shown that tumor rejection can require migratory and resident DCs to sense type I IFNs for the initiation of early tumor responses and to enhance cross-presentation of tumor antigens to $CD8^+$ T cells [59, 60].

Conventional T cells

T cells are crucial players in the adaptive, cellular immune response. Originating from hematopoietic stem cells in the bone marrow, conventional T cells migrate and mature in the thymus, where they somatically rearrange gene segments of the α- and the β-chain forming the TCR. Rearrangement requires enzymes encoded by the recombination-activating genes (*Rags*). There are two *Rag* gene products, RAG-1 and RAG-2, which are exclusively expressed in lymphocytes. Thus, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ mice are devoid of all lymphocytes, including T cells, NKT cells and γδ T cells and also B cells, representing the humoral part of the adaptive immune response [61, 62]. Accordingly, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ have been shown to have increased susceptibility to multiple induced tumors, such as MCA-induced sarcomas [41].

The past years were marked by technical advances in flow cytometry and cell characterization methods which unveiled the complexity and plasticity of a growing number of T cell subsets. For simplification purpose, this thesis will refer to two major subsets distinguished by CD4 and CD8 lineage marker expression. Identifying them as either $CD4^+$ helper T cells (Th) or cytotoxic $CD8^+$ T cells (CTLs), $CD4^+$ T cells recognize immunogenic peptides presented in the context of MHCII, while cytotoxic $CD8^+$ T cells recognize immunogenic peptides in the context of MHCI.

CTLs may apply three distinct mechanisms of killing target cells. Two of these rely on cell-cell contact between the effector and the target cell. Firstly, like NK cells, CTLs can release lytic granules containing perforin and granzymes into the target cell, inducing its apoptosis. Secondly, cell-cell induced apoptosis can be triggered by engagement of Fas

ligand expressed on CTLs and Fas receptor (CD95) expressed on the target cell. The third, cell-to-cell contact independent mechanism involves cytokine production by CTLs. Cytokines such as IFN-γ and TNF-α are being produced following T cell activation. TNF-α can bind to its corresponding receptor expressed on target cells, inducing target-cell apoptosis. In contrast, IFN-γ upregulates expression of MHCI on target cells, increasing recognition by CTLs. Moreover, IFN-γ increases Fas receptor expression on target cells, facilitating Fas-mediated apoptosis [63].

$CD4^+$ Th cells can be classified into a minimum of four classes, Th1, Th2, Th17 and regulatory T cells (Tregs) (Th17 will not be further described here; Tregs are described in section 5.2.1.3). Critical for orchestrating the adaptive immune response, Th1 and Th2 subsets can be distinguished according to cytokine production profile and function. Th1 cells are characterized by IFN-γ and IL-2 production and are responsible for mediating the immune response against intracellular pathogens, promoting cell-mediated immunity. As described above, IFN-γ is involved in shaping the immune response in multiple ways, such as in the activation of macrophages, while production of IL-2 is important for $CD4^+$ T cell memory formation and as a stimulus for the activation of CTLs [64]. Th2 cells are involved in mediating the immune response against extracellular parasites, promoting humoral immunity. Associated with facilitating tumor growth, counteracting Th1 immunity, Th2 cells produce the cytokines IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 [64].

According to the importance of T cells in tumor elimination, mice lacking αβ T cells (*Tcrb*$^{-/-}$) display an increased tumor susceptibility of MCA-induced sarcomas [53]. Moreover, the capacity of T cells to recognize specific antigens, using tumor-specific antigens for both $CD4^+$ and $CD8^+$ T cell, has been exploited successfully in multiple preclinical tumor models and is a strategy for personalized immunotherapy of human cancers [65-68]. Aiming to expand pre-existing neoantigen-specific T cell populations and to induce a broad repertoire of T cell specificity in cancer patients, clinical trials using peptide vaccinations are currently ongoing across multiple types of cancer. For instance, a clinical trial is investigating a peptide vaccine containing the neoepitope IDH1 in grade III-IV glioma patients that harbor the *IDH1R132H*-mutation (NCT02454634) [69, 70].

5.2.1.2. Equilibrium

Cancer cell variants not eliminated can enter the equilibrium phase. During this phase, the immune system exerts sufficient pressure to prevent outgrowth of tumor cells. Tumor

control is mainly mediated by T cells, IL-12 and IFN-γ [33, 69]. In contrast, NK cells and molecules related to recognition and effector function of the innate arm of immunity are not required, indicating that prevention of tumor outgrowth involves adaptive immunity only. Notably, in patients, the equilibrium phase can last for decades before residual tumor cells enter the final phase of escape [33].

Experimental evidence for the existence of the equilibrium phase came from experiments with a mouse model of primary chemical carcinogenesis [71]. In this study, Koebel *et al.* injected immunocompetent mice subcutaneously (s.c.) with the carcinogen MCA. Even though these mice harbored occult cancer cells, they did not develop apparent tumors until the immune system was compromised through depletion of T cells and IFN-γ [71]. Isolated tumor cells were highly immunogenic, resembling unedited tumor cells from MCA-injected immunodeficient *Rag2*$^{-/-}$ mice. Notably, $CD4^+$ and $CD8^+$ T cells, IL-12 and IFN-γ, but not cells of the innate immune system were required to prevent tumor outgrowth. This observation mechanistically distinguished the elimination and equilibrium phase, since the elimination phase requires both innate and adaptive immunity [71].

5.2.1.3. Escape

The escape phase is characterized by the failure of the immune system to eliminate or control transformed cells, resulting in cancer progression, and eventually, a clinical malignancy. Due to genetic and epigenetic changes combined with constant immune selection pressure, some tumor cell variants can develop mechanisms to grow in an immunologically unrestricted manner, circumventing innate and adaptive immunity. This process can lead to the emergence of unusually aggressive tumor variants suppressing the anti-tumor immune response, for example by loss-of-function of genes involved in the MHCI antigen presentation machinery [43]. Thus, mechanisms involved in tumor cell escape can be divided into alterations on the tumor cell level, also referred to as intrinsic mechanisms, and changes affecting the immune response due to tumor-induced immunosuppression also referred to as extrinsic mechanisms including tumor-promoting inflammation.

Intrinsic mechanisms

At the tumor cell level, reduced immune recognition and avoidance of immune destruction can lead to tumor escape. Reduced immune recognition can result from loss of MHC components or defects in the antigen processing function preventing the production of peptide and loading to MHC molecules, required for T cell recognition [33, 72]. Specifically, loss of TAP1, MHCI molecules, β2m, LMP2 and LMP7 favors tumor progression [43]. However, also the loss of tumor-specific antigens due to genomic instability can prevent detection by antigen-specific $CD8^+$ T cells. Alternatively, shedding of NKG2D ligands has been shown to severely compromise the anti-tumor immune response leading to immune evasion in individuals with cancer [73]. However, especially the development of IFN-γ insensitivity during tumor development has been identified as a critical determinant for prevention of T cell-mediated killing and tumor progression. In this respect, the study from Kaplan *et al.* showed complete unresponsiveness to IFN-γ in 25% of human lung adenocarcinoma cell lines [40]. Moreover, defects in the pathways of IFN-receptor signaling have been associated with resistance to PD-1 blockade immunotherapy [74].

Mechanisms leading to escape from immune destruction can also be a result of defects in death-receptor signaling pathways. Resistance to lysis by immune cells can be caused by expression of mutated inactive forms of death receptors, such as the TRAIL receptor DR5 or Fas [43, 75]. In this respect, studies have identified several mutations in TRAIL receptors in samples from patients with metastatic breast cancer [75]. Additional mechanisms leading to escape from immune destruction are related to the expression of antiapoptotic signals. For example, constitutive activation of the pro-oncogenic transcription factor STAT3 has been identified to increase tumor cell proliferation, survival, and invasion [76]. Alternatively, anti-apoptotic mechanisms induced by expression of the cell death inhibiting oncoprotein Bcl-2 promote tumor growth by evading immune destruction.

Tumor-promoting inflammation

In addition to alterations affecting tumor cells themselves, escape can result from the generation of an immunosuppressive tumor microenvironment. Tumor-promoting inflammation, an enabling characteristic of the hallmark of cancer includes the production of cytokines like VEGF, transforming growth factor-β (TGF-β) and indoleamine 2,3-

dioxygenase (IDO) [33]. Notably, VEGF is not only crucial for angiogenesis but is also associated with preventing endogenous DC function. While the highly pleiotropic cytokine TGF-β acts as a tumor suppressor in early stages of tumor proliferation, late-stage expression of TGF-β has been associated with higher tumor grade and poorer prognosis [77]. Known to polarize T cells and cells of the monocyte lineage towards an immunosuppressive state, TGF-β favors tumor growth by promoting angiogenesis and has additionally been associated with sustaining cancer stem cell populations [77, 78]. The cytosolic enzyme IDO, produced by macrophages and dendritic cells in response to pro-inflammatory factors, is responsible for catalyzing tryptophan degradation producing metabolites like kynurenine [77]. Kynurenine is an immunosuppressive factor associated with inhibition of T cell effector functions and supports the expansion of regulatory T cell (Treg) populations suppressing the immune response [79].

Recruitment of immunosuppressive cells represents another critical factor in dampening anti-tumor immune responses, associated with poorer prognosis. Tregs, $CD4^+$ T cells constitutively expressing CD25 and the transcription factors forkhead box (Fox)p3, produce immunosuppressive such as cytokines IL-10 and TGF-β upon stimulation. By expressing negative co-stimulatory molecules like CTLA-4, PD-1, and PD-L1, Tregs actively contribute to an immunosuppressive microenvironment. Moreover, consumption of IL-2 by Tregs, a cytokine required for the maintenance of cytotoxic T cell function, severely impacts the anti-tumor response [33]. Another immunosuppressive cell type involved in supporting tumor growth are myeloid-derived suppressor cells (MDSCs). MDSCs represent a heterogeneous group of myeloid progenitor and immature myeloid cells that induce Tregs and produce multiple immunosuppressive cytokines including TGF-β [80]. Moreover, MDSCs have been implicated in consuming amino acids like arginine, tryptophan, and cysteine, essential for proper T cell function [33]. An additional myeloid-derived cell type supporting immunosuppression are M2-polarized tumor-associated macrophages (TAMs). Recruited from peripheral blood, chemokines and growth factors induce the differentiation of monocytes into M2-polarized macrophages. Factors promoting polarization include cytokines produced by tumor cells (IL-4, IL-10), and cytokines produced by cells of the immune system, like Treg-derived IL-10 and Th2-derived IL-4/IL-13. TAMs contribute to immunosuppression with the production of TGF-β and IL-10 [81, 82].

In summary, the immune system plays a dual role in cancer by suppressing tumor growth and selecting tumor cells more fit to evade immune detection. How a tumor-specific immune response is generated will be described in the following section.

5.2.2. The cancer-immunity cycle

Generating an anti-tumor immune response is a multistep process also referred to as the cancer-immunity cycle (Figure 4).

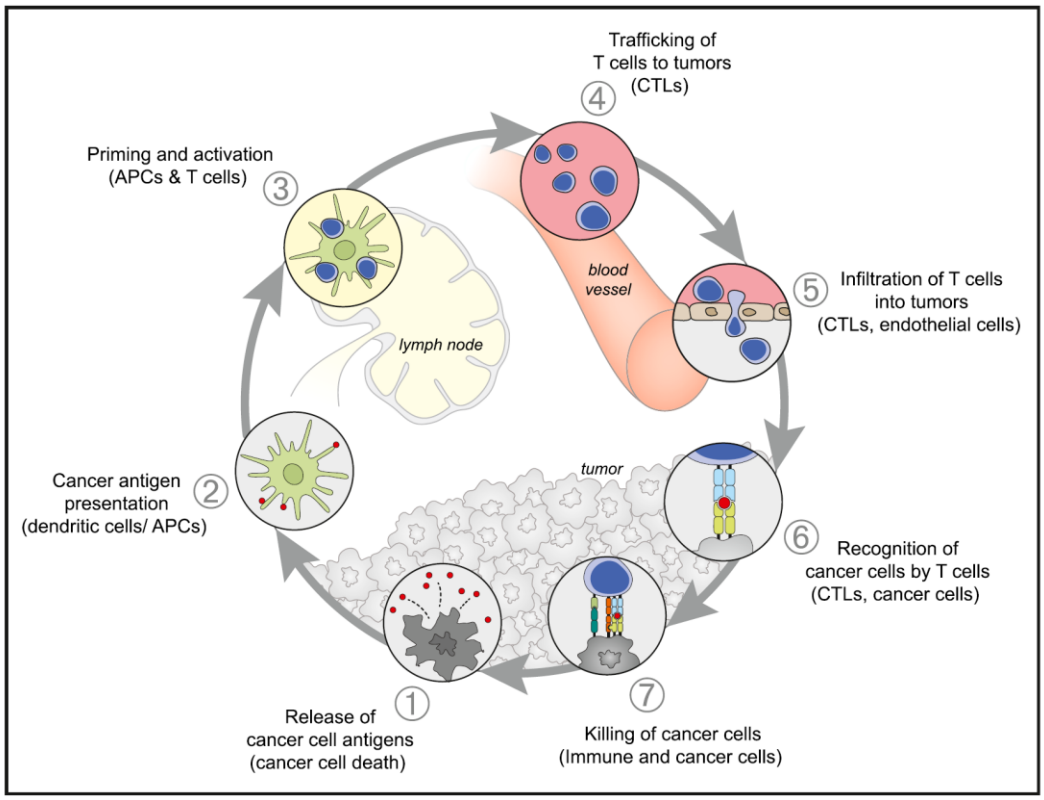

Figure 4: **The cancer-immunity cycle.** The development of an adaptive immune response is a multistep process divided into seven steps including antigen release, presentation, priming and activation of T cells, trafficking of T cells to tumors, infiltration, recognition, and killing of cancer cells. Adapted from [83].

Beginning with the release of neoantigens upon cancer cell death, DCs capture and process the derived neoantigens (Step 1). During step 2, DCs present captured antigens on MHC molecules and migrate to the tumor-draining lymph node. Alternatively, direct antigen drainage can occur, leading to neoantigen capture and presentation within the tumor-draining lymph node. Presentation of cancer-antigens then induces priming and activation of T cells (Step 3). Specifically, activation, expansion, and differentiation require the incorporation of three independent signals. Signal one consists of immunogenic peptides presented by MHC molecules to the cognate TCR. During signal

2, the immunological synapse stabilizes through the interaction of costimulatory molecules including but not limited to CD80/CD86 expressed on APCs and CD28 on T cells. Signal 3 includes secretion of cytokines by APCs, polarizing T cells towards an effector phenotype. IL-12 secreted by APCs induces polarization of Th1 cells, while IL-4 promotes Th2 differentiation.

In Step 4, T cells traffic through blood vessels and infiltrate the tumor bed (Step 5). Upon recognition of cancer cells, CTLs kill the target cancer cell. The killing of cancer cells inducing the release of more neoantigens, which continues the cancer-immunity cycle [83].

5.2.2.1. The immunity cycle in glioma

Given the regulation of immune cell entry into the CNS by the blood-brain barrier, the absence of a conventional lymphatic drainage system and overall low numbers of APCs and T cells, the CNS has long been viewed as an immune-privileged site. However, it has become increasingly clear that the CNS undergoes constant immune surveillance, being capable of orchestrating immune responses involving both innate and adaptive immunity [84]. With the discovery of a lymphatic system draining CNS antigens by the cerebrospinal fluid into cervical lymph nodes, it is now believed that both egress and entry are dynamically regulated [77, 85]. Especially in neurological diseases, immune cells readily migrate into the CNS following chemotactic cues. In glioma, the interaction of the tumor and immune cells is additionally facilitated with increased VEGF secreted by tumor cells, inducing permeability of the blood-brain barrier [84].

Regarding the cancer-immunity cycle described for glioma, antigens from dying tumor cells can be processed by infiltrating macrophages, DCs and CNS-resident microglia. Even though microglia can actively contribute to an anti-tumor immune response through the release of reactive oxygen species, phagocytosis, presenting antigens and activating T cells, they have been described to lose the capacity of expressing MHC molecules in high-grade gliomas [77]. In this respect, within the glioma-immunity cycle, DCs are believed to migrate through the lymphatic vessels of the meningeal sinuses, priming and activating T cells in the cervical lymph nodes. Activated T cells then traffic to the tumor site interacting with APCs and tumor cells by migrating through the blood-brain barrier and the blood-tumor barrier [84].

However, as described in section 5.2.1.3, tumors exploit multiple mechanisms of immune suppression to evade immune destruction. Especially malignant gliomas are characterized by inducing profound immunosuppression, both locally and systemically [86].

Concluding, the cancer-immunity provides multiple targets to boost the anti-tumor immune response. Examples of current immunotherapy-based strategies aiming to overcome glioma-induced immunosuppression will be outlined in section 5.3.3.

5.3. Malignant glioma

Multiple types of tumors have been identified in the CNS. Among them, malignant gliomas represent the most common primary CNS tumor with an incidence of 6 new cases per 100´000 individuals per year worldwide [87]. Based on data collected from the years 2012-2014, the lifetime risk of developing brain and other nervous system cancer has been estimated to be approximately 0.6% for men and women [88].

Even though gliomas can arise from distinct cell types including neural stem cells, astrocytes, or oligodendroglial progenitor cells, the majority of gliomas are astrocyte-derived [89]. Roughly 50% of newly diagnosed gliomas are classified as glioblastoma, a highly malignant brain cancer associated with a median survival of 14-17 months despite aggressive treatment consisting of surgical resection, radiotherapy, and chemotherapy.

The only established environmental risk factor remains ionizing radiation [90]. However, multiple familial cancer predisposition syndromes have been associated with increased risk of brain cancer. Among them is neurofibromatosis type I (caused by mutations in *NF1* or *NF2*), the Turcot syndrome (mutations in genes associated with DNA repair) and the Li Fraumeni syndrome (mutations in checkpoint gene *TP53*) [87, 91]. Additionally, polymorphisms in the 3′-untranslated region of *IL12A* resulting in lower IL-12 production has been correlated with an increased susceptibility to develop glioblastomas [92].

Non-specific initial symptoms are primarily due to increased intracranial pressure caused by tumor growth and include headaches, nausea, and vomiting [93]. Specific symptoms depend on the location and encompass seizures (occurring in 25% of patients with high-grade gliomas), partial weakness of one side of the body or difficulties in memory [24, 90]. The gold standard for diagnosis is magnetic resonance imaging (MRI), however, also computed tomography (CT) provides aid in defining the surgical strategy. Thus far, no serum markers have been identified allowing early detection [87].

One of the reasons for poor survival is the invasive, diffuse infiltration pattern into surrounding tissue, resulting in the inability of complete surgical resection [94]. Additionally, tumor heterogeneity contributes to the failure of conventional therapies. Analysis of glioblastoma patient samples before and post-treatment revealed variable degrees of genetic similarity and acquired mutations typical for recurrent tumors. The capacity of developing therapy resistance has been attributed to cancer stem cells, also referred to as recurrence-initiating stem-like cancer cells [95]. Several studies supported the notion of stem-like cancer cells being crucial for resistance to therapy [96, 97]. However, markers defining these cells remain a subject of debate.

5.3.1. Classification and molecular characterization

Based on the "WHO Classification of Tumors of the CNS" from 2007 [98], gliomas were previously exclusively classified according to histological criteria, grouping tumors per origin of cell type as astrocytoma, oligodendroglioma or ependymal tumors [94]. Additionally, each tumor received a histological grade, reflecting the degree of malignancy ranging from slow-growing, surgically curable lesions (WHO grade I) to highly malignant, diffusely infiltrating tumors (WHO grade IV). However, especially in the context of diffusely infiltrating tumors, this classification system led to considerable inter-observer variations [99]. Thus, the revised "WHO Classification of Tumors of the CNS" from 2016 included molecular characteristics, enabling a more precise tumor characterization and prognosis, as depicted in Figure 5 [100].

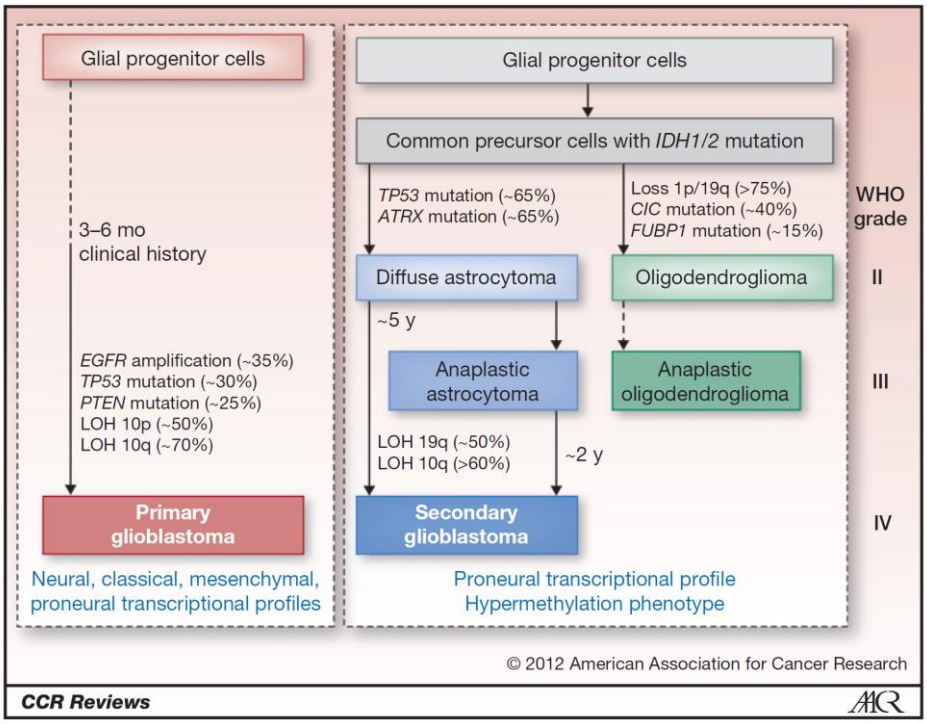

Figure 5: **Genetic pathways in primary and secondary glioblastomas.** Adapted from [101].

The diagnostic biomarker distinguishing gliomas with distinct biology and clinical behavior is based on the presence of mutations in the isocitrate dehydrogenases (*IDH*) 1 or 2, discriminating between primary and secondary glioblastomas. Primary glioblastomas develop *de novo* and have a short clinical history of 3-6 months before diagnosis. Associated with *TP53* and *PTEN* mutations, this particularly aggressive tumor is characterized by significant proliferation, invasion, angiogenesis and is refractory to treatment with survival rates of 14-17 months after diagnosis [102]. In contrast, secondary glioblastomas develop progressively from WHO grade II tumors. Roughly 90% of WHO grade II tumors harbor *IDH1/2* mutations, causing aberrant DNA and histone methylation [12]. In this respect, mutated *IDH1* has been associated with induction of hypoxia-inducible factor, glucose transporters and increased VEGF production leading to the hallmarks of altered energy metabolism and increased angiogenesis [103]. However, additional molecular characterization is required to distinguish oligodendrogliomas (codeletion of 1p/19q) and diffuse astrocytoma (*TP53* mutations), the latter ultimately progressing to treatment-refractory WHO grade IV secondary glioblastomas. Notably, WHO grade II tumors are not surgically curable anymore and are characterized by proliferation and invasion. Even though considered as treatment responsive, patients

diagnosed with WHO grade II astrocytomas have a median survival of 5-10 years [102]. Within five years, anaplastic astrocytoma (WHO grade III) develop. In addition to increased proliferation and invasion, WHO grade III astrocytomas have acquired features of angiogenesis. Despite lower survival rates of 2-3 years, grade III astrocytoma remain treatment responsive. Nevertheless, within two years, anaplastic astrocytoma can develop into treatment refractory, secondary glioblastomas (WHO grade IV).

Predictive biomarkers for therapy include O^6-methylguanine-methyltransferase (*MGMT*) promoter methylation. The *MGMT* gene encodes for a DNA repair protein, which removes alkyl products from the O^6 position of guanine. Epigenetic silencing leading to loss of gene transcription and reduced protein expression has been correlated with increased sensitivity to alkylating chemotherapeutics like temozolomide (TMZ). Notably, 75% of secondary glioblastoma and 36% of primary glioblastoma (IDH wild-type) are characterized by this promoter methylation [104].

5.3.2. Current treatment for glioma

The current standard of care for gliomas includes surgery, radiotherapy, and chemotherapy. Decisions in treatments are based on tissue diagnosis and identification of molecular markers, the age of the patient and Karnofsky performance (classifying patients based on functional impairment). Additionally, before surgery patients frequently receive corticosteroids, especially dexamethasone, to decrease tumor-associated edema [84, 87]. Surgeries aim for maximal safe resection using tools such as surgical navigation systems with functional MRI datasets and the fluorescent dye 5-aminolevulinic acid to visualize tumor tissue. Electromyography in patients under local anesthesia is performed when tumors reside in areas where resection may cause permanent neurological deficits [87].

Radiotherapy as a backbone of standard treatment after surgery is used in the treatment of newly diagnosed and recurrent glioblastoma and aims to improve local tumor control and overall survival. However, even though improvement of survival rates has been shown for every tumor grade, no patients have been cured with this treatment modality [94]. Due to the unmasking of tumor antigens induced by radiotherapy, it has been suggested that radiotherapy combined with immune checkpoint inhibition might have a synergistic effect. Supported by preclinical results [105], currently running clinical trials will show whether the combination of radiotherapy and immune checkpoint inhibition will also improve patient survival (see in section 5.3.3) [84].

Together with radiotherapy, cytotoxic chemotherapy using the DNA alkylating agent TMZ represents the gold standard of treatment for most patients with glioma. As described in section 5.3.1, it requires assessment of *MGMT*-promotor methylation as a predictive biomarker, since tumor cells expressing low to absent MGMT levels are more sensitive to TMZ treatment [106]. However, tumors with *MGMT*-promotor methylation can acquire mutation-driven secondary TMZ resistance [107]. Dose-limiting toxicity of TMZ results from myelosuppression, especially leukopenia and lymphopenia and might cause treatment delay, reduction or initiation of alternative treatments [84]. Other chemotherapeutic treatments use nitrosourea-based compounds, such as carmustine. Local delivery of carmustine wafers (Gliadel®) implanted into the surgical cavity has previously been approved by the FDA for intracerebral chemotherapy of malignant glioma [108]. However, leading to modest survival advantages and poor efficacy with newly diagnosed WHO grade III, IV, or recurrent gliomas, carmustine wafers are rarely considered for therapies nowadays [87].

In cases of progressive disease despite prior therapy, patients can be treated with bevacizumab, an antibody inhibiting the activity of VEGF. However, this anti-angiogenic drug has only been approved in some countries, for instance, the United States and Switzerland, since lack of a controlled trial prevented approval in the European Union [109].

5.3.3. Prospects of immunotherapeutic approaches

Despite current standard of care, glioblastoma ultimately relapses in almost all patients [84]. No standard treatment can currently prolong survival in these patients, underlining the unmet need for continuous development of therapies. Due to the success of immunotherapy with immune checkpoint inhibitors for the treatment of other tumor types, immunotherapy has become an appealing strategy for glioblastoma [84, 110-115]. Justified by several promising preclinical datasets, multiple studies have been performed investigating distinct molecules and combinations.

Studies activating co-stimulatory receptors (e.g., OX-40) or blocking of co-inhibitory receptors (e.g., PD-1 and CTLA-4) have been shown to induce anti-tumor responses leading to prolonged survival in distinct preclinical glioma models [84, 105, 116, 117]. Moreover, combinations aiming to overcome immunosuppression and to increase effector to T_{reg} ratio in the tumor microenvironment have led to long-term survival. Among the

combinations evaluated are triple therapies consisting of IDO, CTLA-4, and PD-L1 [118], combinations of immune checkpoint inhibition with radiotherapy [105, 119] and the combination of local pro-inflammatory cytokine release with CTLA-4 blockade [120]. As 88% of newly diagnosed glioblastoma patient samples and 72% of recurrent glioblastoma patient samples stain positive for PD-L1, this led to the initiation of many clinical trials blocking this axis, as exemplified in Table 1 [84, 121-123]. Unfortunately, no results are available from these studies so far, but the PD1/PD-L1 axis may play a prominent role in glioblastoma.

Other strategies to revert the glioma-associated immunosuppression into a potent anti-tumor immune response rely on vaccine-based immunotherapeutic approaches. Target antigens may be predefined tumor-associated antigens, a personalized panel of tumor-associated antigens, an unbiased antigen selection using undefined tumor-derived peptides or whole tumor cell lysates [124]. Alternatively, vaccines can target tumor-specific mutant proteins like the epidermal growth factor receptor (EGFR) variant III (EGFRvIII), which is frequently amplified in IDH wild-type glioblastomas [124-126]. Initial promising results were obtained in three uncontrolled phase II trials leading to progression-free and overall survival improvement for patients with resected tumors without progression after chemoradiotherapy [124, 127-129]. However, a later conducted phase III trial with newly diagnosed glioblastoma trial (ACT-IV) had to be terminated since no improvement of overall survival was achieved [130].

Based on current knowledge, overcoming the glioma-associated immunosuppression remains a significant obstacle and likely requires the combination of several immunomodulatory agents. In this regard, further results from preclinical studies will support decisions on which combinations can increase therapeutic activity.

Table 1: **Overview of initiated clinical trials for primary brain tumors and brain metastases targeting the PD-1/PD-L1 axis.** (Registered on clinicaltrials.gov; status 18/09/2017) [131, 132].

Registration number	Agents tested	Therapy	Tumor	Phase	Status
NCT02374242	anti-PD-1, anti-CTLA-4	Nivolumab + Ipilimumab	Melanoma; Brain Metastases	II	Active, not recruiting
NCT02621515	anti-PD-1, anti-CTLA-4	Nivolumab + Ipilimumab	Melanoma; Brain Metastases	II	Recruiting
NCT02978404	anti-PD-1	Nivolumab + Radiosurgery	Metastatic Renal Cell C arcinoma; Non-Small Cell Lung Cancer Meta-static; Brain Metastases	II	Recruiting
NCT02669914	anti-PD-L1	Durvalumab	Brain Metastases from Epithelial-Derived Tumors	II	Recruiting
NCT01952769	anti-PD-1	Pidilizumab	Diffuse pontine gliomas	I + II	Active, not recruiting
NCT02529072	anti-PD-1	Nivolumab + Surgery + DC Vaccination	Malignant Glioma; Astrocytoma; Glioblastoma	I	Recruiting
NCT02658981	anti-LAG3 + anti-PD-1	Urelumab + Nivolumab	Glioblastoma; Gliosarcoma; Recurrent Brain Neopla sm	I	Recruiting
NCT02852655	anti-PD-1	Pembrolizumab	Recurrent/Progressive Glioblastoma		Active, not recruiting
NCT02335918	anti-CD27 + anti-PD-1	Varlilumab + Nivolumab	Glioblastoma	II	Not recruiting
NCT03058289	anti-PD-1	INT230-6 + anti-PD-1 antibody	Melanoma; Head and Neck Cancer; Lymphoma; Breast Cancer; Pancreatic Cancer; Liver Cancer; Colon Cancer; Lung Cancer; Glioblastoma	I + II	Recruiting
NCT01860638	anti-VEGF	Bevacizumab + Lomustine + Radiotherapy + Temozolomide	Glioblastoma	II	Completed
NCT03174197	Anti-PD-L1	Atezolizumab + Radiotherapy + Temozolomide	Newly diagnosed glioblastoma	I + II	Recruiting

5.3.4. Experimental glioma models

Choosing appropriate rodent models is crucial for studying treatment approaches. An ideal glioma model should combine several features including the recapitulation of human disease characteristics of invasion and angiogenesis, the possibility of genetic modification, being reproducible and having predictable growth patterns [133]. Currently, several preclinical glioma models are being used to investigate the interaction of tumor cells with the CNS and infiltrating immune cells. Classified into xenograft, allograft, and spontaneous transgenic models, examples of each will be described below.

One of the frequently used xenograft glioma models is the U251 malignant glioma cell line originally derived from a 75-year-old male with glioblastoma [134]. Injected intracranially into SCID mice, this mouse model recapitulates characteristics of glioblastoma such as the infiltrative pattern of invasion into normal brain parenchyma [135]. Histochemical analysis showed similarities to human glioblastoma, as tumor cells were positive for glial fibrillary acidic protein (GFAP), vimentin and S100B [135-138]. Genetically, the U251 cell line has been shown to possess key features resembling human glioblastoma, for instance, a dysfunctional mutant tumor suppressor *TP53* and deletions in the cell cycle suppressor genes $p14^{ARF}$ and *p16* [135, 138]. A significant drawback, however, in using xenograft models like the U251 malignant glioma model is that it does not recapitulate the interaction of immune cells with tumor cells of human glioblastoma, arguing for a shift away from xenograft models for immunotherapy approaches.

Allograft models used as preclinical glioma models can be induced by chemical induction to generate a stable transplantable cell line such as the astrocyte-derived GL-261 cell line [139] (further described in section 5.3.4.1) or derived from spontaneously developing tumors. A frequently used cell line from a spontaneously developed glioma derives from the VM mouse strain obtained through homogenization of the tumor tissue and several *in vitro* and *in vivo* passages [135, 140]. Resembling anaplastic astrocytoma, the derived SMA-560 cell line is characterized by low S-100 expression and high GFAP expression [135, 141]. Moreover, SMA-560 cells express TGF-β, an immunosuppressive factor secreted in human glioblastoma [142]. Notably, the SMA-560 tumor model has been used for several studies of immunotherapy applications. For instance, genetically modified T cells expressing chimeric antigen receptors binding to the EGFRvIII led to abrogation of tumor growth and conferred immunological memory [143].

In addition to allograft and xenograft glioma models, several transgenic models leading to spontaneous glioma development have been generated. The first transgenic astrocytoma model was developed in 1995, inducing expression of the pro-tumorigenic SV40 large T antigen in astrocytes under the control of the GFAP promoter [144]. Other transgenic mouse models were generated taking key genetic alterations involved in primary and secondary glioblastoma initiation or progression into account, such as deficiency in the tumor suppressor TP53. In this respect, deficiency in TP53 specifically in astrocytes promotes the formation of high-grade gliomas in mice that simultaneously express the $HRas^{V12}$ oncogene [145-147]. However, whether the interaction between immune cells and tumor cells is recapitulated in these mice remains to be investigated.

5.3.4.1. The GL-261 glioma model

One of the most commonly used rodent glioma models in the context of immunotherapy studies for the treatment of glioblastoma is the orthotopic GL-261 tumor model. This astrocyte-derived GL-261 tumor cell line was initially made through intracranial injections of MCA into the brains of mice [139] and is a syngeneic mouse model in C57BL/6 mice.

GL-261 tumor cells carry distinct oncogenic alterations in genes associated with human carcinogenesis, such as increased expression of the *Myc* oncogene and inactivation of the tumor suppressor *p53* [135, 148]. Moreover, the GL-261 cell line carries a point mutation in the *K-Ras* gene [149].

Additionally, GL-261 tumor cells have been characterized to express basal levels of MHCI, which can be increased with IFN-γ [149]. Moreover, IFN-γ can also induce MHCII expression. In contrast, overexpression of other cytokines did not affect MHCI or MHCII expression [120, 149]. Furthermore, GL-261 tumor cells express basal levels of the costimulatory molecules CD80 and CD86, classifying GL-261 tumor cells as moderately immunogenic [150].

Tumor antigens identified include the murine homolog AN2 of the human melanoma proteoglycan and the glioma antigen GARC-1 for cytotoxic lymphocytes [150-152]. Additionally, it has been shown that GL-261 tumor cells express the shared melanoma antigens glycoprotein (gp)100, tyrosinase-related protein 2 (TRP-2) and the receptor tyrosine kinase EphA-2 [153, 154].

Unlike spontaneous glioma, GL-261 tumor cells display a radial growth pattern and upon injection, tumor cells form small islets scattered around the injection site [150]. Injected into the right striatum, 100 cells were found to be sufficient for mice to reach withdrawal criteria within 70 days [149]. However, for studies using the GL-261 tumor model, the number of injected cells varies between 1 x 10^4 and 13 x 10^4 cells [105, 120].

Immunotherapy approaches in the GL-261 tumor model

Multiple approaches have been investigated with the GL-261 tumor model. These include adoptive T cell transfers, monoclonal antibodies directed against relevant immunological targets such as CTLA-4, active immunotherapy with dendritic cells loaded with tumor antigens, gene therapy using genetically modified tumor cells together with *in situ* gene therapy and studies involving overexpression of cytokines [150].

Regarding adoptive transfer setups, 30 million TILs together with systemic administration of IL-2 and local tumor irradiation led to tumor rejection in 15% of treated animals [155]. In contrast, sub-lethal whole body irradiation combined with 10 million total effector T cells led to a complete rescue of mice [156].

Treatment approaches using monoclonal antibodies for boosting the anti-tumor immune responses have been shown by enhancing co-stimulatory signaling through OX-40 and 41-BB [150]. Moreover, blockade of CTLA-4 in combination with IL-12 significantly improved the anti-tumor immune response leading to 80% of tumor rejection [120]. Also, systemic inhibition of TGF-β combined with peptide-based vaccination against known glioma-associated antigen led to the prolonged survival of mice [157]. Other approaches targeting the immune checkpoint surface receptor PD-1, in combination with radiation led to 25% survival of mice [105].

Vaccination experiments were performed by several groups using whole cell, whole tumor antigen, and antigen-specific approaches. Notably, preventive treatment leading to 50% of protection of tumor growth in mice was achieved by loading DCs with total GL-261 RNA lysate, underlining the immunogenicity of the GL-261 cell line [158]. Whereas in established GL-261 tumors, DCs fused with tumor cells, local radiation and systemic treatment with anti-OX40 antibodies were required to induce protection against glioma [159]. Moreover, the requirement of Treg elimination for successful treatment of mice with tumor lysate-pulsed DCs has been previously established [160]. Vaccinations with

the immunodominant peptides gp100 and TRP-2 were found to be sufficient to induce tumor protection in 80% of mice [153].

Gene therapy approaches aiming to increase the immunogenicity of tumor cells by inducing overexpression of distinct cytokines have mainly focused on the cytokines GM-CF, IL-2 and IL-12 (preclinical models used with IL-12 overexpressing tumor cells will be described in section 5.4.2 and 5.4.2.1). In this respect, mice treated with IL-2 secreting fibroblasts and oral treatment with the chemotherapeutic pioglitazone prolonged survival of mice [161]. Immunization with tumor cells overexpressing GM-CSF improved survival of mice and was found to synergize with whole body irradiation leading to 40-80% tumor protection [162, 163].

In summary, the GL-261 tumor model has been used extensively for the investigation of immunotherapy approaches, leading to several advances in experimental immunotherapy for the treatment of glioblastoma.

5.4. The IL-12 cytokine family

The IL-12 cytokine family consists of the four heterodimeric cytokines IL-12, IL-23, IL-27, and IL-35. One of the distinctive features of the IL-12 cytokine family is the chain sharing of cytokines and receptors. However, despite structural similarities, cytokines, and receptors of the IL-12 family shape the outcome of immune responses in distinct ways, having both pro-inflammatory and inhibitory effects (Figure 6) [164].

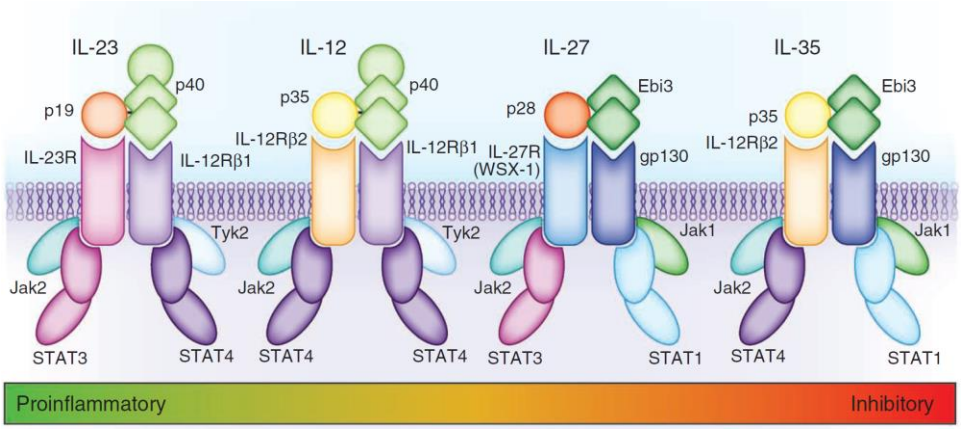

Figure 6**: The IL-12 cytokine family.** Cytokines with their respective receptor subunits and associated JAK-STAT signaling. Adapted from [164].

The pro-inflammatory cytokine IL-12, identified in 1989 as a natural killer cell stimulatory factor, has a molecular weight of 70kDA and consists of the disulfide-linked

light p35 subunit and the heavy p40 subunit [165]. While the p40 subunit is expressed in abundance, the p35 subunit is constitutively expressed at low levels [166]. However, only co-expression of both subunits can induce biologically active IL-12. Binding of IL-12 to the IL-12Rβ1 and IL12Rβ2 subunits generates Th1 responses by signaling through STAT4 (IL-12 mechanism of action will be described in section 5.4.1) [167].

IL-23 shares the p40 subunit with IL-12 but forms the functional IL-23 cytokine together with the disulfide-linked p19 subunit. IL-23 produced by DCs, monocytes, macrophages and B cells is known to act on memory T cells, NK cells, Th1 and Th17 expressing the functional receptor composed of the IL-12Rβ1 and IL-23R subunits. Signaling through STAT3 and STAT4, IL-23 signaling is involved in Th1 activation and Th17 polarization and activation [164].

The IL-12 family member IL-27 consists of the subunits p28 and the Epstein-Barr virus-induced gene 3 (EBI3). Major sources of IL-27 are macrophages, inflammatory monocytes, and DCs, while plasma cells, endothelial cells, and epithelial cells are considered as minor sources of IL-27 [168, 169]. Expression of IL-27 has been associated with the resolution phase of an autoimmune response [164, 170, 171]. The functional IL-27 receptor is composed of gp130 and the IL-27Rα (WSX-1), expressed by NK cells, $CD4^+$ and $CD8^+$ T cells. Signaling through STAT1 and STAT3, IL-27 inhibits the development of Th17 cells and promotes IL-10 producing regulatory T cells. Together with IL-12, IL-27 can support the production of IFN-γ by T and NK cells, supporting pro-inflammatory responses [164]. Due to its context-dependent influence on both pro-and anti-inflammatory immune reactions, IL-27 is thus considered to have an immunoregulatory role [164].

IL-35 is composed of the subunits p35 and EBI3, while the receptor has been suggested to consist out of the IL12Rβ2 and gp130 or IL27Rα subunits [164]. Produced by Tregs, IL-35 has been proposed to inhibit T cell responses and to convert naïve T cells into Tregs by signaling through STAT1 and STAT4 [172]. However, with a recently retracted publication regarding Tregs requiring IL-35 for immune suppression [173], the induced mechanisms of IL-35 remain a subject of debate.

5.4.1. Mechanism of IL-12 action

Produced by antigen presenting cells such as dendritic cells, IL-12 can activate and induce proliferation of IL-12 receptor expressing ILCs, NKT, and T cells. Thus, IL-12 has been

attributed to link innate and adaptive immune responses. IL-12 signaling further leads to polarization of a Th1 immune response, suppressing Th2 immunity. The primary cytokine produced upon IL-12 signaling is IFN-γ, which acts on APCs and induces increased expression of IL-12. However, IL-12 may also trigger the release of other cytokines such as TNF-α, GM-CSF, and IL-2 [166]. Importantly, IL-12 signaling is known to induce cytotoxicity mediated by the release of lytic granules containing perforin and granzymes into a target cell. Moreover, IL-12 can have an anti-angiogenic effect by triggering the production of interferon-inducible protein 10 (IP-10), an inhibitor of neovascularization [174].

Apart from the established IL-12 receptor expression on ILCs, NKT and T cells, cells of myeloid cells origin and tonsillar B cells have previously been shown to express the IL-12 receptor [175, 176]. In an experimental model of vesicular stomatitis virus (VSV) infection of the CNS, enhanced recovery has been found to be mediated by neurons expressing the IL-12 receptor [177]. In this respect, also microglia have been shown to express the IL-12 receptor in primary culture conditions upon stimulation with IL-12 [178, 179]. Moreover, IL-12 signaling in keratinocytes has been shown to induce a tissue-protective response in the Aldara-induced psoriatic plaque formation model [180].

5.4.2. IL-12 in preclinical tumor models

The tumor-protective role of IL-12 has been established for both the cytokine and the receptor. Mice lacking the p35 subunit display increased numbers of chemically induced papillomas and increased incidence of N-methyl-N-nitrosourea-induce T cell lymphomas [166, 181, 182], while the lack of the p40 subunit led to accelerated growth of MCA-induced sarcomas [183]. The absence of IL12Rβ2 subunit expression has been shown to predispose to malignancy increasing the incidence of spontaneous tumors and accelerating the growth of transplantable tumors [184].

Preclinical models investigating the anti-tumor immune response induced by IL-12 include the B16 melanoma model, the CT26 colon carcinoma model, the TSA mammary carcinoma model and the 4T1 breast cancer model amongst others (IL-12 in preclinical glioma models will be described in section 5.4.2.1). However, its mechanism of action has been shown to differ among tumor models. Factors influencing tumor-rejection are dose, timing, and location of IL-12 release/injection. In the B16 melanoma model, overexpression of IL-12 in tumor cells led to tumor suppression mediated by a subset of

Rorγt-dependent innate lymphoid cells [185, 186]. While other studies using established s.c. B16 tumors and i.p. injection of IL-12 led to a $CD8^+$ T cell-dependent tumor rejection [187]. In contrast, increasing the dose of IL-12 for the treatment of established B16 tumors required NK and NKT cell-dependent mechanisms of tumor suppression [183, 188].

The transplantable BALB/c colon carcinoma CT26 model has also been extensively used within IL-12 treatment approaches. However, IL-12 given systemically did not have any effect on the s.c. injected primary tumor, most likely due to the absence of responding cell types [189]. In contrast, it has been shown that IL-12 significantly reduces liver metastasis, a mechanism most likely involving liver-resident NKT cells [190]. When CT26 tumor cells were modified to overexpress IL-12, inhibition of tumor take and tumor rejection were dependent on the level of IL-12 expression. Amounts of IL-12 in the pg range led to delayed tumor onset and reduced number of lung metastasis upon s.c. and i.v. injection [189]. However, tumors were only rejected when simultaneous depletion of $CD4^+$ T cells was performed, presumably due to IL-12 receptor expression on regulatory T cells [191]. In contrast, amounts of IL-12 released by CT26 in the ng range did not form tumors, unless injected cell numbers were increased to 10-50 fold of the minimal lethal dose of the parental CT26 cell line [189]. Interestingly, this mechanism was found to be IFN-γ independent but required GM-CSF production by $CD4^+$ T cells for tumor rejection [192].

The TSA cell line, initially derived from a spontaneous mouse mammary carcinoma, has been modified to overexpress distinct cytokines, amongst which IL-12 was found to be the most potent in inducing tumor protection [193, 194]. This mechanism was dependent on cytotoxic $CD8^+$ T cells secreting IFN-γ [195]. In the 4T1 tumor model, considered to be a less immunogenic model for breast cancer, IL-12 did not affect the primary tumor but induced a significant reduction of lung metastasis. This mechanism was found to be partially dependent on IFN-γ-producing NK cells [166, 196-198].

5.4.2.1. IL-12 in preclinical glioma models

Regarding preclinical models for glioma, IL-12 mediated glioma rejection has been claimed to be T cell and NK cell-dependent [120, 199-205]. Strategies in delivering IL-12 include systemic administration, intratumoral delivery, viral transfer systems and cells overexpressing IL-12.

Early studies with a GL-26 glioma model and intratumoral administration using adenoviral delivery of IL-12 at day four post tumor cell injection led to the survival of 50% of mice accompanied by the infiltration of $CD8^+$ and $CD4^+$ T cells [199]. However, these studies lacked functional experiments showing the necessity of T cells for the anti-tumor immune response [199]. Injection of neural stem cells expressing IL-12 on day two post tumor cell injection only led to tumor rejection in 20% of mice. Here, IL-12 led to an influx of T cells. However, this study also lacked functional data to undermine the requirement of T cells for tumor rejection [200]. In another C57BL/6 model, mice were injected i.c. with 203 glioma cells. In this study, treatment consisted of systemic injection of IL-12 and IL-18, together with vaccination of dendritic cells pulsed with Semliki Forest virus (SFV) and 203 glioma cDNA [201]. Here, tumor rejection was induced in a T and NK cell-dependent fashion and required IFN-γ [201].

In the GL-261 tumor model, an approach of using concentrated DNA/PPC (polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol) complexes delivering a murine plasmid encoding IL-12 (pmIL-12) in combination with biodegradable carmustine (BCNU) chemotherapy led to survival in 40% of mice [202]. Moreover, Vetter *et al.* used the GL261 glioma cell model and injected cells into the cerebellum of transgenic mice that constitutively expressed IL-12 under the control of the GFAP promoter in astrocytes, leading to mainly $CD8^+$ T cell-dependent glioma rejection [203]. Our lab has previously shown that local IL-12 delivery combined with systemic blockade of the co-inhibitory receptor CTLA-4 in the GL-261 tumor model leads to tumor rejection in 80% of mice when initiating treatment at day 21 post tumor cell injection. Moreover, tumor rejection was T cell- and perforin-dependent and elicited immunological memory [120].

In studies using a recombinant adeno-associated virus (rAAV) as a vehicle for local delivery of IL-12 in athymic mice bearing DBTRG gliomas, tumor rejection was found to be NK cell-dependent [204]. In a follow-up study, the mechanism was further complemented in that tumor rejection required the activation of microglia expressing TRAIL [205]. Another recent approach used oncolytic herpes simplex virus (HSV) delivering angiostatin (G47Δ-mAngio) and IL-12 (G47Δ-mIL12). Angiostatin, a potent inducer of tumor vasculature regression co-delivered with IL-12, significantly prolonged survival of mice bearing U87 gliomas [206]. While a recent study implicated the importance of macrophages in a triple treatment setup of anti-CTLA-4, anti-PD-1 and

G47Δ-mIL12 in a glioblastoma stem-like cell model (GSC005), leading to tumor rejection in 50% of mice [207].

In summary, the anti-tumorigenic effects of IL-12 are not only tissue-specific but are also time- and dose-dependent, leading to the involvement of distinct responsive effector cell subsets.

5.4.3. IL-12 for the treatment of human cancer

The success of IL-12 obtained in preclinical tumor models prompted the translation into clinical settings. The applied approaches for the treatment of human cancer with IL-12 can be divided into three distinct groups. Firstly, administrating IL-12 alone or in combination with chemotherapy or monoclonal antibodies. Secondly, vaccine-based approaches based on tumor antigen-derived peptides in combination with IL-12. Thirdly, gene delivery systems and adoptive cell transfer systems [208].

Most of the studies performed between 1996 and 2005 aimed to assess safety evaluations of IL-12 and were based on active, non-specific IL-12 therapy. Administered either i.v. or s.c., patients with distinct cancers, such as metastatic renal carcinoma or melanoma, were treated with IL-12 only or in combination with other treatment approaches [166, 209-217]. Promising results were obtained in hematological cancers such as refractory non-Hodgkin`s B-cell lymphoma when IL-12 was combined with rituximab, having 11 complete responders and 18 partial responders out of 43 participants [218]. For solid cancers, however, response rates were low throughout different types of cancers using IL-12 alone but also when used in combination with other treatment approaches. For instance, a complete response was achieved in only one out of 12 enrolled melanoma patients injected i.v. with IL-12 [209]. Moreover, only one out of 51 advanced renal cell carcinoma patients injected s.c. with IL-12 displayed a complete response, while 34 patients had stable and 14 patients progressive disease [213]. Combinatorial approaches for patients with metastatic $Her2^{+}$ breast carcinoma using i.v. and s.c. injected IL-12 together with the chemotherapeutic paclitaxel and trastuzumab resulted in only one complete responder out of 21 enrolled patients [217]. Importantly, not only the low response rates but the occurrence of adverse side effects due to toxicity leading to fatalities abrogated the pursuit of systemic IL-12 therapy [209, 216].

After years of discouragement, there has been a regain of interest for the administration of IL-12 for cancer treatment with the focus on local instead of systemic delivery, aiming

to minimize toxicity while maximizing efficiency [166, 209, 210, 216]. In this respect, there has been an increase in registered clinical trials since 2011 on clinicaltrials.gov, using local delivery systems for IL-12 [208]. Strategies for local delivery include nanoparticle-based systems, immuno-cytokines (IL-12 fused to tumor targeting antibodies), viral delivery and gene therapy approaches [219-225]. Moreover, another approach being investigated uses chimeric antigen receptor (CAR)-modified T cells engineered with the IL-12 gene [208, 226].

Currently registered clinical trials mainly focus on gene delivery systems, e.g., using *in situ* electroporation of plasmids encoding for IL-12 in different cancer types. In this respect, the clinical trials treating melanoma, cutaneous T-cell lymphomas and Merkel cell carcinoma patients have been completed. However, results are not available yet ([166], NCT01502293, NCT01440816, NCT01579318). Studies using the immuno-cytokine NHS-IL12 (construct composed of two IL-12 heterodimers fused to the NHS76 antibody recognizing single-and double-stranded DNA and thereby necrotic areas of the tumor [227]) are still recruiting.

Regarding glioma, three clinical trials using IL-12 therapy are currently registered with clinicaltrial.gov (status 07/09/2017). A study treating high-grade glioma based on dendritic cells expressing IL-12 loaded with autologous tumor lysate in combination with standard therapy (surgery, temozolomide, and radiotherapy) has recently been completed (NCT01213407, [228-230]). The other two registered clinical trials use viral delivery systems, either an engineered HSV-1 expressing IL-12 (NCT0206282, recruiting patients) or an adenovirus vector engineered to express IL-12 (NCT02026271). First results of the latter trial, treating patients diagnosed with recurrent or progressive glioma with a combination of an adenoviral vector delivering IL-12 under the control of an orally given activator ligand (veledimex) were presented at the ASCO 2016 meeting. Peripheral blood samples had an increase of $CD8^+$ T cells and an increased ratio of effector-to-suppressor T cells ($CD8^+$/$FoxP3^+$). Moreover, patients displayed minimal but manageable neurotoxicity [231].

In summary, multiple clinical trials for the treatment of human cancers with IL-12 are currently ongoing. Given the toxicity-related issues in early clinical trials, current strategies aim to minimize toxicity, while maximizing efficacy with intratumoral administration instead of systemic delivery of IL-12.

6. Aim of the study

Glioblastoma is the most malignant type of brain cancer with patients having a median survival of 15-17 months, despite therapy consisting of maximal surgery, radiotherapy, and chemotherapy. The poor prognosis of this cancer type underlines the unmet medical need driving the development of new treatments. Given the success of immunotherapies in other tumor types where conventional therapies provided limited success, there is considerable interest in developing immunotherapies for the treatment of glioblastoma.
We previously showed that immunomodulation by reverting the suppressive tumor microenvironment into a pro-inflammatory microenvironment can lead to glioma rejection [120]. Using the C57BL/6 syngeneic GL-261 glioma model, with GL-261 tumor cells continuously releasing the proinflammatory cytokine IL-12 and firefly (*Photinus pyralis*) luciferase for monitoring tumor development by bioluminescence imaging, we identified that IL-12 mediated glioma rejection involves T cells, but not NK cells, is perforin-dependent and IFN-γ-independent. However, translation of this knowledge into the human situation for the development of new therapies requires an immunological understanding of the mechanistic underpinnings, including the characterization of directly and indirectly affected cells. Thus, the objective of this study was to investigate the mechanism of IL-12 in triggering tumor rejection by studying the contribution of:

1.) the CNS-resident cells versus hematopoietic-derived cells regarding their requirement of responding to IL-12

2.) IL-12 in the context of the glioma immune-cycle by studying the contribution of intratumoral versus lymph node immune responses, including tracking of tumor-specific T cells and investigating the involved antigen presenting cell types.

7. Disclaimer

This thesis was based upon and partly adapted from the following manuscript:

” IL-12 mediated glioma rejection relies on local responsiveness of $CD8^+$ T cells”

Vrohlings M, Tugues S, Haftmann C, Leung B, Sassi A, Jaberg J, Becher B. manuscript in preparation.

8. Materials and methods

Animals. C57BL/6 mice were obtained from Janvier, and congenic C57BL/6-CD45.1 and CD45.1/2 bred in-house; *ccr2*$^{-/-}$ on a C57BL/6 background were kindly provided by L. Borsig (University of Zurich, Zurich, Switzerland). *IL12rb2*$^{-/-}$, *Rag1*$^{-/-}$ mice were purchased from Jackson Laboratories. OT1 and OT2 mice bred on the C57BL/6 background were kindly provided by M. van den Broek (University of Zurich, Zurich, Switzerland) and the Laboratory Animal Science Center (LASC) of the University of Zurich. *Batf3*$^{-/-}$ mice were kindly provided by M. Suter (University of Zurich, Zurich, Switzerland).

In most experiments, mice at 7-12 weeks of age were used. All animals were kept in-house consistent with institutional guidelines under specific pathogen-free conditions with food and water provided ad libitum at a 12-h light/dark cycle. All experiments were performed according to institutional guidelines and approved by the Swiss cantonal veterinary office (licenses 65/2012; 25/2015).

Murine tumor cell lines. All cell lines (GL-261luc:Fc, GL-261luc:IL12Fc and GL-261luc:cOva) were maintained at 37°C, 10% CO2 in DMEM supplemented with 10% FBS, 1%P/S. As previously described, GL-261 tumor cells were modified to continuously release a fusion protein of the proinflammatory cytokine IL-12 joined with the crystallizable fragment of IgG3 (IL12Fc) or only the IgG3 (Fc) fragment as a control. Moreover, cells were modified to express Photinus pyralis luciferase (GL-261luc) for monitoring tumor bioluminescence imaging (BLI) and kept under antibiotic selection [120].

The generation of GL-261luc with stable expression of chicken ovalbumin (GL-261luc:cOva) was induced by lentiviral transduction. In brief, lentiviral particles were generated by transfecting 293T cells with the packaging constructs psPAX2 and pVSV (a kind gift from L. Wong, University of Zurich, Zurich, Switzerland) and the relevant lentiviral plasmid. The chicken ovalbumin sequence was cloned into the pLenti CMV Blast DEST backbone (Eric Campeau (Addgene plasmid # 17451). The complete construct pLenti CMV Blast DEST cOVA was a kind gift from M. van den Broek. GL-261luc cells expressing chicken ovalbumin were kept under antibiotic selection using blasticidin (invivogen).

Stable production of IL-12Fc was achieved by lentiviral transduction with the plasmid SFG IL12 IL12Fc I2 dCD8 (a kind gift from Sergio Quezada, University College London, London). Cytokine production was detected by ELISA (OptEIA mouse IL-12/23p40; BD).

Bone marrow chimera. For the generation of bone marrow chimeras, donor mice were killed by CO_2 inhalation. Femur, tibia, radius, and hipbones were removed and flushed with PBS to isolate bone marrow cells. Cells were then passed through a 70 μm pore size filter and washed with PBS. Recipient mice were lethally irradiated with 1,100 rads (split dose) and received 1 x 10^6 to 5 x 10^6 bone marrow cells. Engraftment took place over 6 to 8 weeks before subjecting mice to orthotopic glioma rejection.

Adoptive transfer. For adoptive transfer experiments, $CD4^+$ T cells (L3T4) or $CD8^+$ T cells (Ly-2) were MACS sorted with positive selection (Miltenyi Biotec). Subsequently, cells were injected i.v. in a 1:1 ratio of $CD4^+$ and $CD8^+$ T cells.

Depletion of $CD4^+$ T cells. On day -1, mice were injected i.p. with 500 μg anti-CD4 (GK1.5; Bio X cell) or IgG2b (MCP-11; BioXCell) and then injected on day 0 with tumor cells as described below. Mice were injected every two weeks with the same amount of antibody. Depletion was confirmed by flow cytometry with an anti-CD4 antibody (RM4-5; Biolegend) on a weekly basis.

Orthotopic glioma inoculation. Inoculation into the right striatum was performed as previously described [120]. In brief, 7-12-week-old mice were i.p. injected with meloxicam (Metacam;1-2mg/kg body weight) before being anesthetized with 3.5 - 4% Isoflurane (Minrad) in an induction chamber. Mice were kept under anesthesia on the stereotactic frame (David Kopf Instruments) by delivering 3 % Isoflurane delivered through a nose adaptor. For tumor cell injections, a syringe (Hamilton; 75N, 26s/2"/2.5 μl; blunt-ended) was positioned at the coordinates 1.5 mm lateral and 1 mm frontal of the bregma. The syringe was lowered by 4 mm and retracted 1 mm to form a small reservoir. A microinjection pump was used to inject 3-5 x 10^4 cells per 2 μl at a rate of 1μl/min (UMP-3; World precision Instruments Inc.). Two minutes after injection ended, the

needle was retracted at a rate of 1mm/min. Bone wax was used to close the site of injection (Aesculap; Braun), and the scalp wound was sealed with tissue glue (Indermil; Henkel).

In vivo bioluminescent imaging. Tumor-bearing mice were injected with D-Luciferin (150mg/kg body weight; Caliper Life Science). Animals were transferred to a dark chamber of a Xenogen IVIS 100 (Caliper Life Sciences) imaging system, and luminescence was recorded. The Living Image 2.5 software (Caliper Life Sciences) was used to analyze the data. A circular region of interest (ROI; 1.46cm diameter) was defined around the tumor site, and photon flux was used to quantify tumor size.

Survival analysis. Tumor-bearing animals were monitored by BLI, checked for neurological symptoms and weighed weekly until day 21 after glioma inoculation. Animals were checked daily from day 21 onwards. Upon showing symptoms such as apathy, severe hunchback posture, or weight loss exceeding 20 %, animals were euthanized by CO2 inhalation.

Cell preparations. Cervical lymph nodes, spleen and the frontal part of the tumor-bearing cerebral hemisphere were harvested, cut into small pieces and incubated in collagenase and DNAse (0.4 mg/ml). Subsequently, samples were passed through a 18G syringe. CNS cell suspensions were enriched by a 30% Percoll gradient (GE Healthcare; 1.3 g/ml). Flow cytometric analysis was carried out as described below.

Flow cytometry. The following antibodies were used for flow cytometry analyses: anti-CD45 (30-F11; BioLegend), anti-CD45.1 (Ly-5.2; BioLegend), anti-CD45.2 (Ly-5.1; Biolegend), anti-CD11b (M1/70; BD), anti-CD4 (RM4-5; GK1.5; BioLegend), anti-CD8 (53-6.7; BioLegend; BD), anti-CD44 (IM7; BioLegend), anti-CD62L (MEL-14; BD), anti-CD11c (N418; BioLegend), anti-Ly6C (AL-21; BD), anti-Ly6G (1A8; BioLegend); anti-Siglec-H (551; eBioscience), anti-CD24 (M1/69; BioLegend; BD), anti-MertK (DS5MMER; eBioscience), anti-CD64 (X54-5/7.1; Biolegend), anti-I-A/I-E-AlexaFlour700 (M5/114.15.2; BioLegend), anti-CD103 (2E7; eBioscience), anti-CD86 (GL-1; BioLegend), anti-Ki67 (SolA15; eBioscience), anti-CD3 (17A2; BD), anti-NK1.1 (PK136; BioLegend), anti-CD19 (1D3; BD), anti-PDCA-1 (eBio129c; eBioscience) anti-TCRVb5.1,5.2 (MR9-4; BioLegend). For the exclusion of dead cells, the Zombie Aqua

fixable viability kit or the Zombie NIR fixable viability kit (BioLegend) was used. Single cells were gated from the FSC-A/FSC-H gate.

For tracking proliferation, MACS-sorted $CD4^+$ and $CD8^+$ T cells were CFSE-labeled according to the manufacturers` protocol (Thermo Fisher Scientific). Intracellular cytokine, transcription factor and Ki67 staining were performed using the eBioscience FoxP3 staining buffer set according to manufacturer's instructions. The acquisition was performed on the FACS Canto II (BD), the LSRII Fortessa flow cytometer (BD) or the FACS Symphony (BD). Data analysis was performed using FlowJo Version 10 (Tree Star), proliferation indices calculated with FlowJo Version 9 (Tree Star).

Statistical analysis. For all non-survival analyses of two experimental groups, an unpaired, two-tailed Student's t-test was performed. For all non-survival analyses of three or more groups, a one-way ANOVA with Tukey`s multiple comparison test was performed. For statistical analysis of Kaplan-Meier survival curves, a Log-rank (Mantel-Cox) test was used to calculate p-values. P-values <0.05 are considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All quantitative analyses were performed with GraphPad Prism version 5.0a for Mac OSX (GraphPad Software, Inc).

9. Results

9.1. Identifying the IL-12 responsive cell subsets required for tumor rejection

9.1.1. Glioma rejection requires IL-12 receptor expression on hematopoietic cells

To systematically investigate the role of brain-resident versus hematopoietic bone marrow (BM)-derived immune cells responsible for the tumor-suppressive effects of IL-12, we generated BM chimeras by transferring BM from C57BL/6 WT into *IL12rb2*$^{-/-}$ mice or vice versa (Figure 7A). Upon challenge with GL-261luc:IL12Fc cells, we found that initial tumor growth was comparable among all groups until day 35 (Figure 7B, right panel). However, from day 35 onwards, mice that lacked IL12-responsive hematopoietic cells displayed accelerated tumor growth and were not able to reject IL-12 expressing glioma cells. In contrast, tumor control and survival were functionally restored in *IL12rb2*$^{-/-}$ mice receiving WT bone marrow cells (Figure 7B).

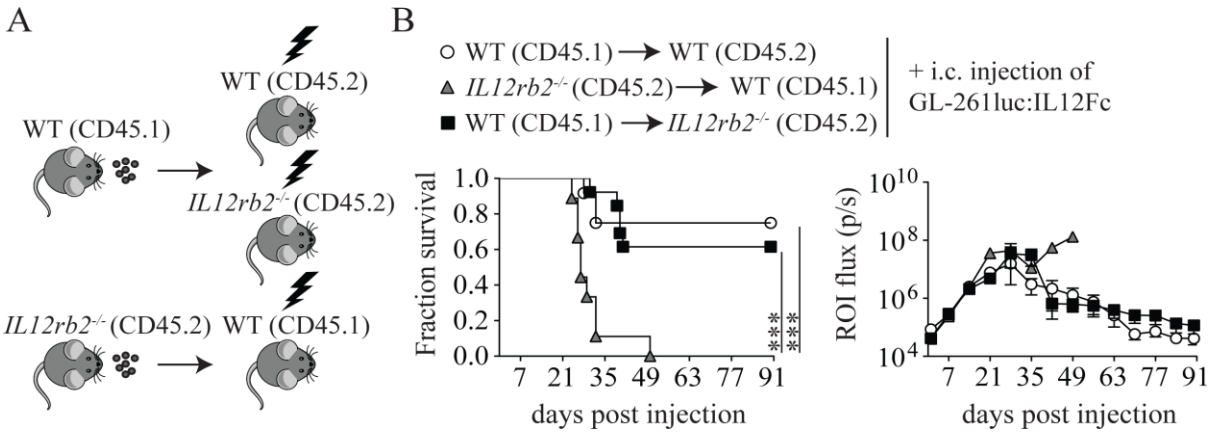

Figure 7: **Glioma rejection requires IL12-receptor expression on BM-derived cells. (A)** Experimental setup for BM chimera using lethally irradiated CD45.1 WT, CD45.2 WT or *IL12rb2*$^{-/-}$ mice reconstituted with CD45.1 WT or *IL12rb2*$^{-/-}$ BM. **(B)** (Left) Survival of BM chimera challenged with GL-261luc:IL12Fc monitored for 90 days. (Right) Tumor development assessed with BLI (photon/s) emitted from the region of interest (ROI) around the tumor site (WT CD45.1 → WT CD45.2; n = 12), (*IL12rb2*$^{-/-}$ → WT CD45.1; n = 9), (WT CD45.1 → *IL12rb2*$^{-/-}$; n = 12). Pooled data from two independent experiments.

In summary, these results indicate that IL-12 tumor rejection relies on BM-derived cells responding to IL-12.

9.1.2. Tumor rejection requires IL-12-receptor signaling in CD8$^+$ T cells

Within the hematopoietic compartment, T cells and innate lymphoid cells represent potential candidate effector populations for IL-12-mediated tumor suppression [120, 166, 183, 185, 187, 189]. Given that NK cells were not required for glioma rejection in our model [120], we investigated the involvement of CD4$^+$ and CD8$^+$ T cells in regards to IL-12 receptor signaling.

To understand whether CD4, CD8 or both T cell subsets are functionally required for IL-12-mediated tumor rejection, we adoptively co-transferred MACS-purified CD4$^+$ and CD8$^+$ T cells into *Rag1*$^{-/-}$ mice using WT or *IL12rb2*$^{-/-}$ donors (Figure 8A). Once T cells expanded, all groups were challenged with IL12Fc tumors. As shown in Figure 8B, a complete responsive T cell compartment and functional IL-12 receptor signaling in CD8$^+$ T cells was sufficient to permit IL-12-driven glioma suppression.

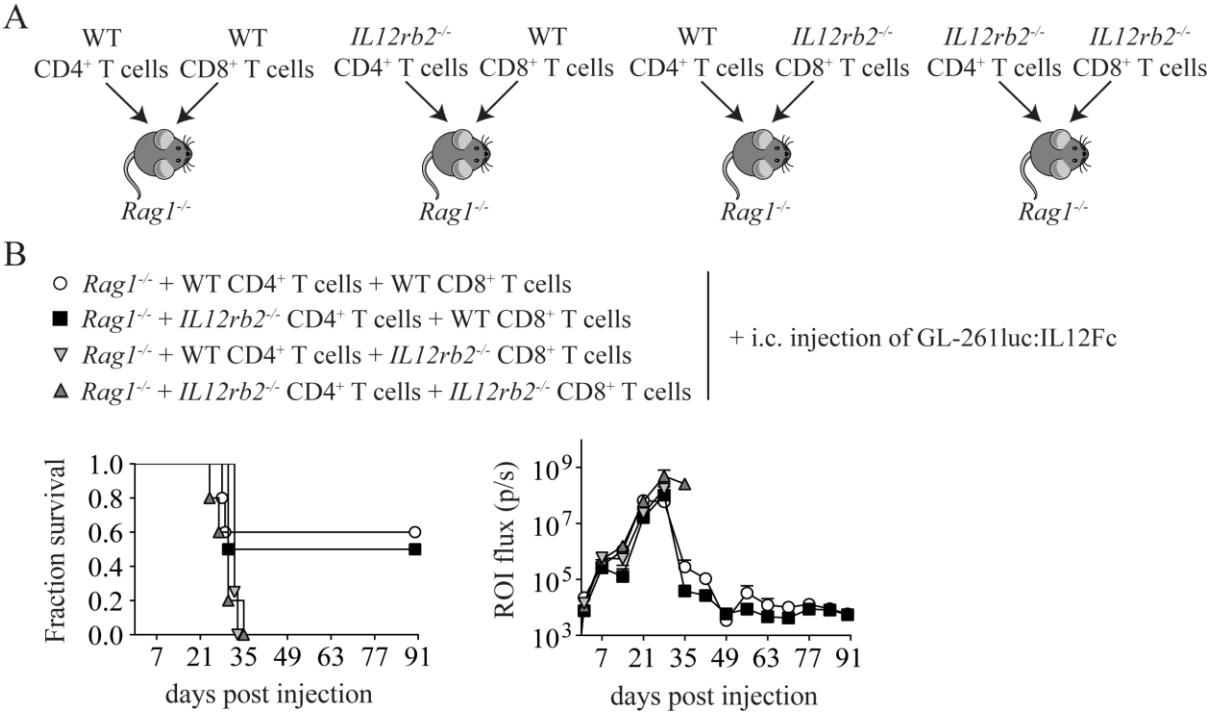

Figure 8: **Glioma rejection requires IL12-receptor signaling in CD8$^+$ T cells. (A)** Experimental setup for the adoptive transfer of T cells into *Rag1*$^{-/-}$ mice. Mice were challenged with IL-12 overexpressing GL-261 tumor cells 17 days after adoptive T cell transfer. **(B)** (Left) Survival of mice monitored for 90 days. (Right) Tumor development quantified with BLI (photon/s) emitted from the ROI around the tumor site (*Rag1*$^{-/-}$ + WT CD4$^+$ T cells + WT CD8$^+$ T cells; n = 5), (*Rag1*$^{-/-}$ + *IL12rb2*$^{-/-}$ CD4$^+$ T cells + WT CD8$^+$ T cells; n = 2), (*Rag1*$^{-/-}$ + WT CD4$^+$ T cells + *IL12rb2*$^{-/-}$ CD8$^+$ T cells; n = 5), (*Rag1*$^{-/-}$ + *IL12rb2*$^{-/-}$ CD4$^+$ T cells + *IL12rb2*$^{-/-}$ CD8$^+$ T cells; n = 4).

Next, we investigated T cell responses in an immune competent microenvironment. Therefore, mixed chimeric mice bearing both WT (CD45.1) and *IL12rb2*$^{-/-}$ or WT

(CD45.1) and WT (CD45.2) hematopoietic compartments were generated to study the competitive response between T cells deficient or functional in IL-12-signaling (Figure 9A). Eight weeks after reconstitution, equal expansion of transferred cells was confirmed, and mice challenged with IL12Fc tumors (Figure 9B).

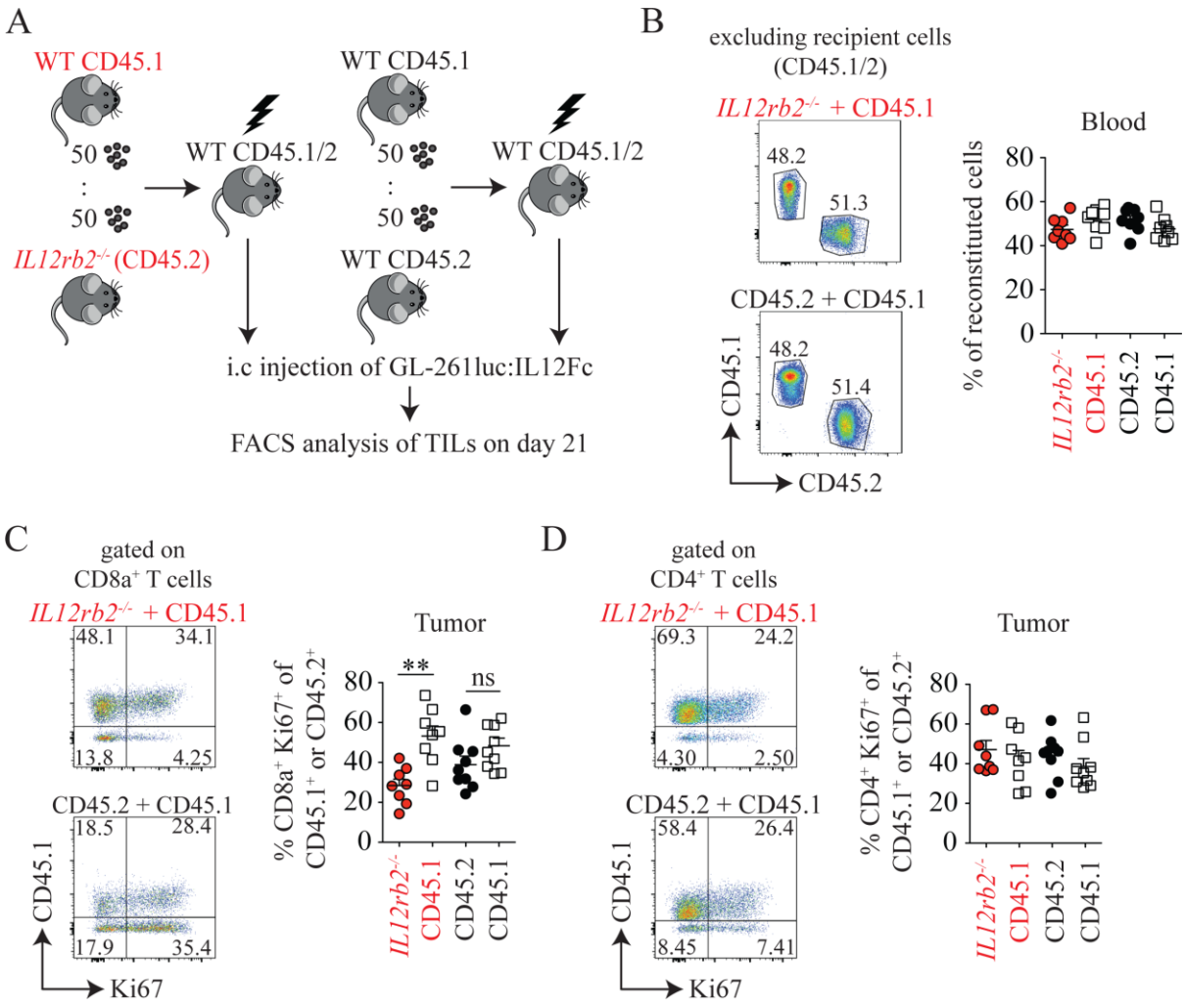

Figure 9: **Local IL-12 release induces proliferation of tumor-infiltrating CD8$^+$, but not of CD4$^+$ T cells. (A)** Experimental setup for mixed bone marrow chimera. **(B)** (Left) Reconstituted mice from mixed BM chimera based on single positive CD45.1$^+$ or CD45.2$^+$ FACS staining in the blood and statistical summary (right). **(C, D)** Tumor-infiltrating CD8$^+$ **(C)** and CD4$^+$ T cells **(D)** on day 21 post i.c. challenge with GL-261luc:IL12Fc cells. (Left) Example FACS staining of Ki67$^+$ T cells and CD45.1$^+$ or CD45.2$^+$ single positive cells. (Right) Statistical summary of the competitive response between the co-transferred populations (*IL12rb2*$^{-/-}$ + CD45.1 → CD45.1/2; n = 8), (CD45.2 + CD45.1 → CD45.1/2; n = 9). Pooled data from two independent experiments.

The analysis of tumor-infiltrating lymphocytes (TILs) on day 21 post-injection showed an increased proliferation of the responsive CD8$^+$ T cell fraction (Figure 9C) compared to CD8$^+$ T cells derived from *IL12rb2*$^{-/-}$ donors, as assessed by Ki67$^+$ staining. In contrast, the proliferative CD4$^+$ T cell response was comparable among all groups (Figure 9D).

Given that IL-12 receptor signaling in $CD4^+$ T cells was dispensable, we next aimed to understand the requirement of $CD4^+$ T cells for IL-12-mediated tumor rejection. Therefore, mice were treated with an anti-CD4 depletion antibody (or the respective isotype control) before and during IL-12 tumor challenge (Figure 10A, B). As depicted in Figure 10C, mice lacking $CD4^+$ T cells did not reject IL-12 expressing tumors.

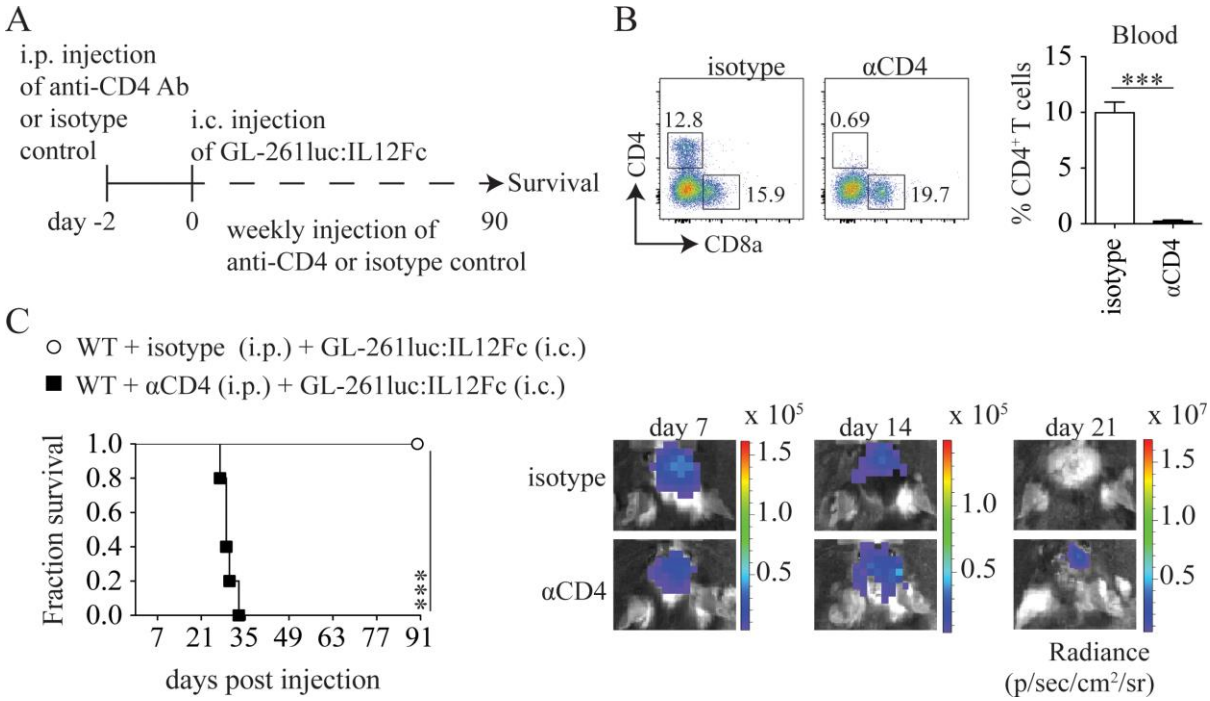

Figure 10: **Glioma rejection requires $CD4^+$ T cell-mediated activation of $CD8^+$ T cells. (A)** Experimental setup for the depletion of $CD4^+$ T cells in WT mice before and during IL-12 tumor challenge. **(B)** Example staining from the blood one day before surgery of mice injected with an anti-CD4 depletion antibody or isotype control. **(C)** (Left) Survival of mice challenged with IL-12 overexpressing GL-261 tumor cells monitored for 90 days (WT + isotype; n = 7), (WT + αCD4; n = 5). (Right) Example of BLI (photon/s) emitted from the ROI around the tumor site on day 7, 14 and 21 post-intracranial injection.

In summary, these data suggest that glioma rejection mainly relies on the local action of IL-12 leading to the proliferation of tumor-infiltrating $CD8^+$ T cells. $CD4^+$ T cells are required for tumor rejection, but dispensable for IL-12 signaling.

9.2. IL-12 mediated glioma rejection in the context of the glioma-immune cycle

9.2.1. Intratumoral versus LN-required immune response during glioma rejection

Since IL-12 induced proliferation of T cells is limited to pre-activated T cells [167], we used the tumor model antigen chicken ovalbumin (cOva) expressed by GL-261 tumor cells (GL-261luc:cOva) to investigate the contribution of IL-12 on tumor-specific T cells during the priming and effector phase of glioma rejection. CFSE-labeled OT1 $CD8^+$ and OT2 $CD4^+$ T cells were adoptively transferred into mice challenged with an equal number of GL-261luc:cOva and GL-261luc:IL12Fc, GL-261luc:cOva and GL-261luc:Fc, GL-261luc:Fc and GL-261luc:IL12Fc or with GL-261luc:Fc cells only (Figure 11A).

By day five post adoptive transfer, the tumor size of the GL-261luc:cOva/IL12Fc and GL-261luc:Fc/IL12Fc the group was reduced compared to that of the GL-261luc:cOva/Fc and the control GL-261luc:Fc group, confirming the tumor-suppressive action of IL-12 in this experimental setup (Figure 11B). We found OT1 (CD45.1/2) $CD8^+$ T cells infiltrating the tumor proliferating in both the GL-261luc:cOva/IL12Fc and the GL-261luc:cOva/Fc groups (Figure 11C). However, the transferred OT1 (CD45.1/2) $CD8^+$ T cells from GL-261luc:cOva/IL12Fc tumors had a clear proliferative advantage as detected by the high peak of the $CFSE^{low}$ fraction and increased division index (Figure 11C, D). We could not detect any proliferation in GL-261luc:Fc/IL12Fc or GL-261luc:Fc tumors, confirming that the IL-12 induced proliferation of T cells is limited to pre-activated, tumor-specific T cells. Regarding the cervical lymph nodes, we only detected a low frequency of proliferating OT1 (CD45.1/2) $CD8^+$ T cells in all groups (Figure 11E, F).

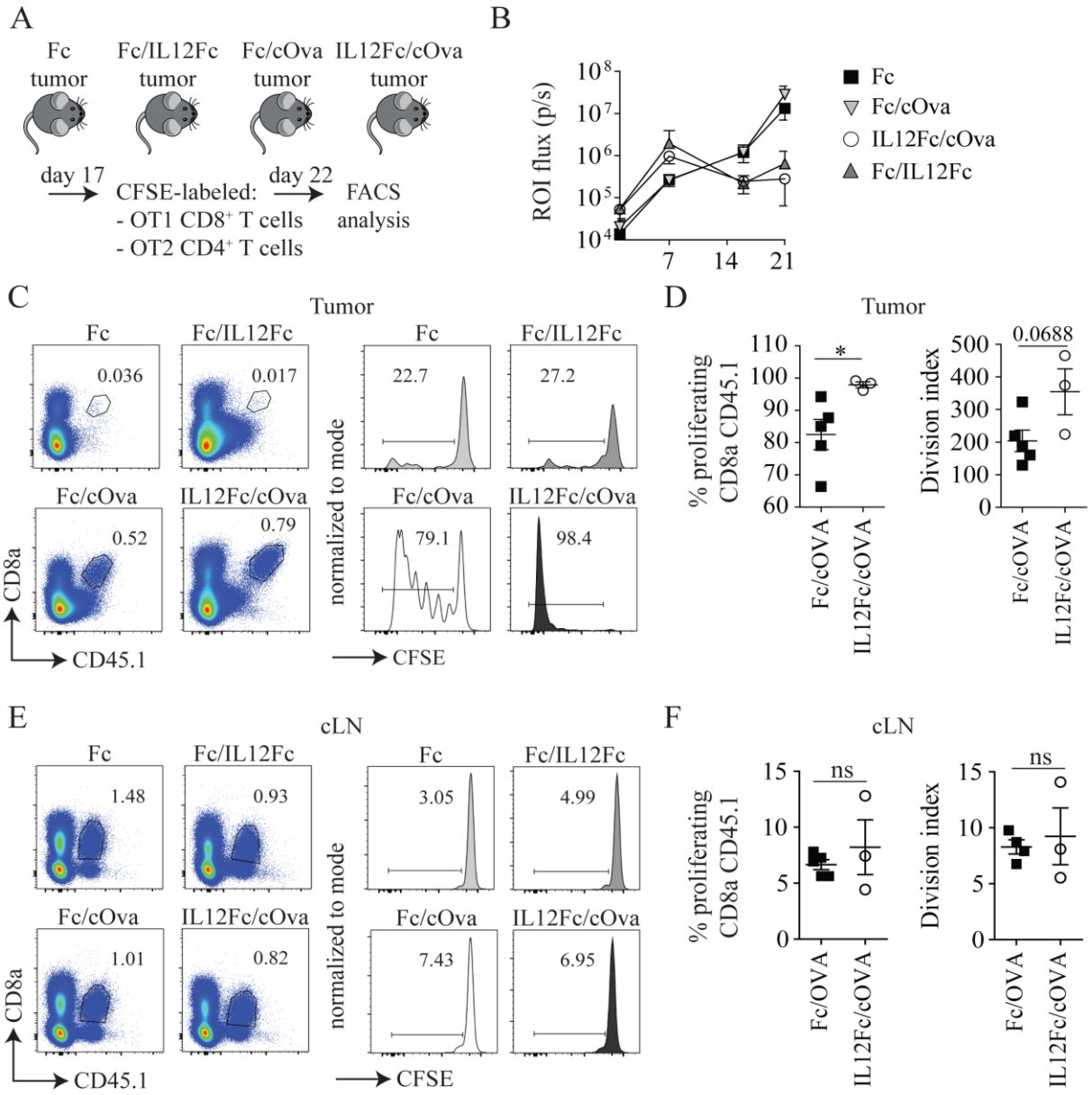

Figure 11: **Local IL-12 induces proliferation of tumor-specific CD8$^+$ T cells within the tumor, but not in the cervical lymph nodes. (A)** Experimental setup for the adoptive transfer of CFSE-labelled OT1 CD8$^+$/OT2 CD4$^+$ tumor-specific T cells into GL-261luc:cOva/IL12Fc, GL-261luc:cOva/Fc, GL-261luc:FcIL12Fc or GL-261luc:Fc tumor bearing WT mice **(B)** BLI measurement on day 7, 16 and 21 after tumor cell inoculation (n ≥ 8/group)). **(C, E)** Tracking of tumor-specific OT1 CD8$^+$ T cells based on the expression of CD45.1 in the tumor and cervical lymph nodes. **(D, F)** Statistical summary (%) of proliferating CD8$^+$ T cells (CFSE$^{low/int}$) and division index in the tumor and cervical lymph nodes. (Fc tumor; n = 5), (Fc/IL12Fc tumor; n = 2), (Fc/cOva tumor; n = 5), (IL12Fc/cOva; n =3). Representative experiment from two independent experiments.

As depicted in Figure 12, we also analyzed the transferred, tumor-specific OT2 (CD45.1/2) CD4$^+$ T cells. Notably, we only detected a minor increase of proliferation of the transferred tumor-specific CD4$^+$ T cells in GL-261luc:cOva/IL12Fc tumors compared to GL-261luc:cOva/Fc tumors (Figure 12A, B). Moreover, we could not detect any

proliferation of OT2 (CD45.1/2) CD4$^+$ T cells in the cervical lymph nodes (Figure 12C, D).

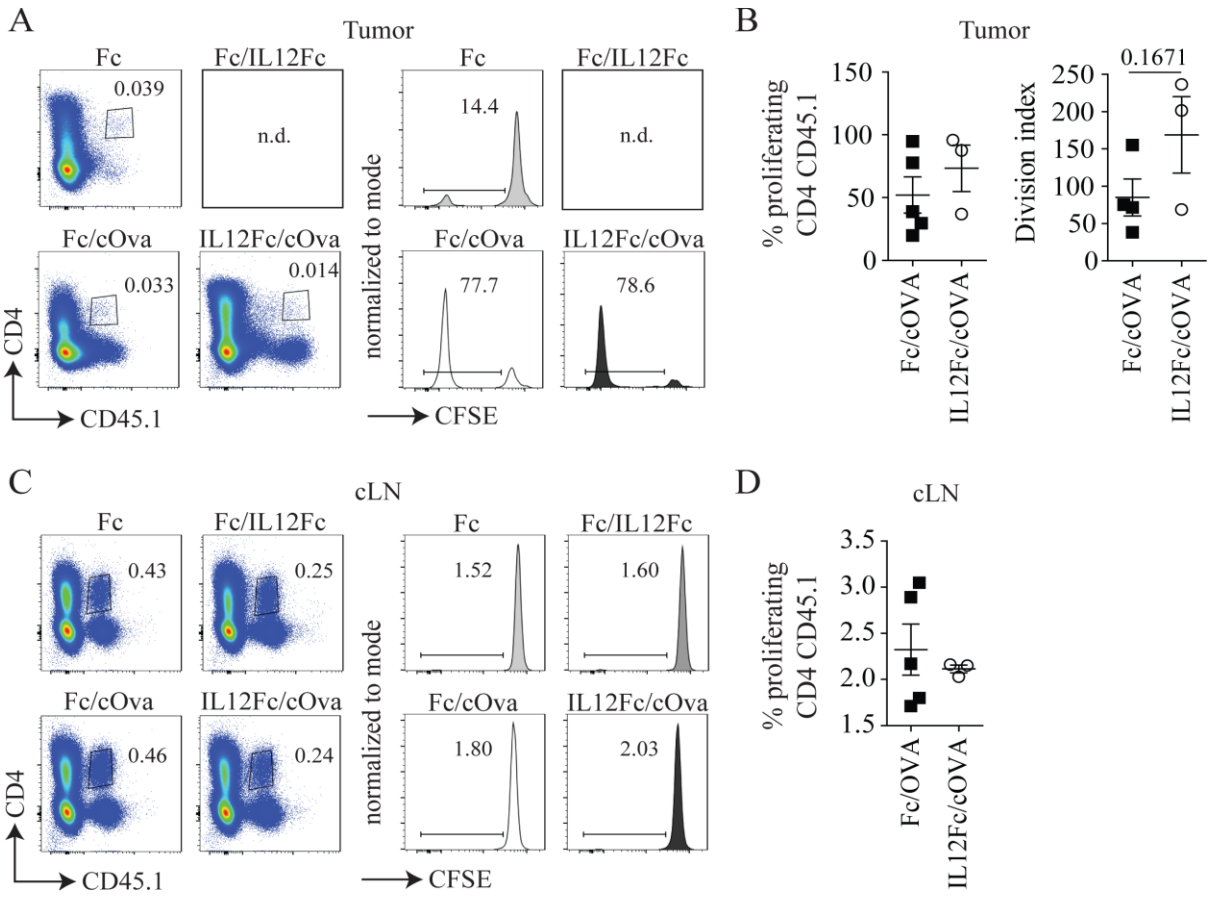

Figure 12: **Local IL-12 does not increase the proliferation of tumor-specific CD4$^+$ T cells in the tumor or cervical lymph nodes. (A, C**) Tracking of tumor-specific OT2 CD4$^+$ T cells based on the expression of CD45.1 in the tumor and cervical lymph nodes. **(B)** Statistical summary (%) of proliferating CD4$^+$ T cells (CFSE$^{low/int}$) and division index within the tumor. **(D)** Statistical summary (%) of proliferating CD4$^+$ T cells (CFSE$^{low/int}$) in the cervical lymph nodes. (Fc tumor; n = 5), (Fc/IL12Fc tumor; n = 2), (Fc/cOva tumor; n = 5), (IL12Fc/cOva; n =3). Representative experiment from two independent experiments.

In summary, these data confirm that IL-12 mainly induces its effect locally by triggering proliferation of tumor-specific CD8$^+$ T cells, while the small population of transferred CD4$^+$ T cells detected at the tumor site displayed low to absent proliferation. Regarding the cervical lymph nodes, there was no increase of proliferation of tumor-specific CD4$^+$ or CD8$^+$ T cells in any of the analyzed groups.

Since we could not detect changes in the proliferative status of tumor-specific T cell in the cervical lymph nodes, we decided to study the role of peripheral versus *in situ* priming during IL-12 mediated glioma rejection. Therefore, we treated mice with the sphingosine-

derivative Fingolimod (FTY720). This immunomodulatory drug prevents lymphocyte egress by activating the S1P receptor 1, subsequently inducing down-regulation of the S1P receptor 1 and thereby retention of lymphocytes within secondary lymphoid organs [232].

To allow initial infiltration of immune cells to the tumor site, we let GL-261luc:IL12Fc and GL-261luc:Fc control tumors grow until day 11 before blocking lymphocyte egress (Figure 13A). As depicted in Figure 13B, FTY720 treatment led to a considerable reduction of T cells in the blood. Moreover, retention of lymphocytes within the secondary lymphoid organs abrogated IL-12 mediated tumor rejection (Figure 13C).

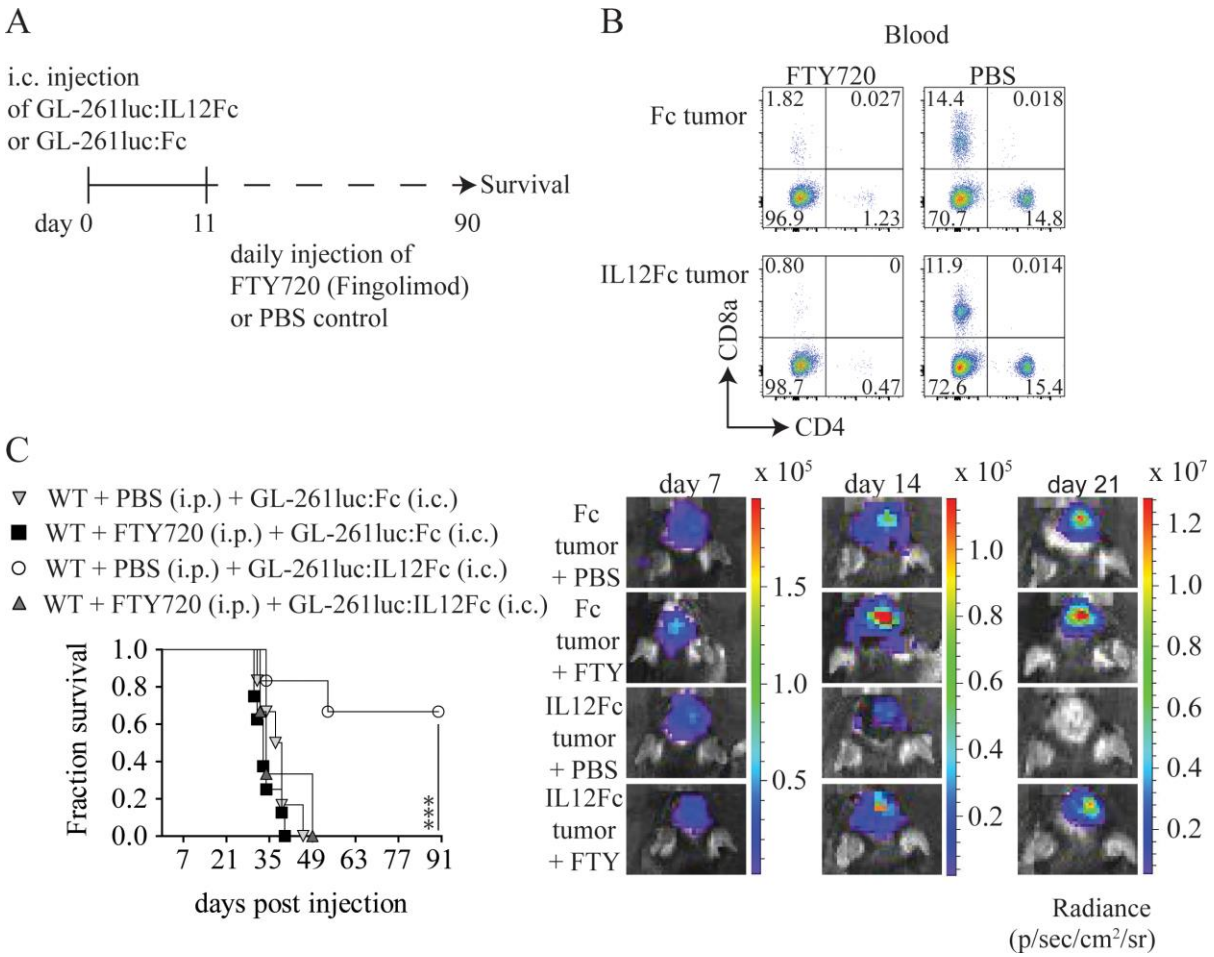

Figure 13: **Glioma rejection requires peripheral priming of T cells. (A)** Experimental setup for blocking lymphocyte egress from secondary lymphoid organs with FTY720. **(B)** Example staining for $CD4^+$ and $CD8^+$ T cells in the blood from mice injected i.p. with FTY720 or PBS. **(C)** (Left) Survival of mice i.c. challenged with GL-261luc:Fc or GL-261luc:IL12Fc monitored for 90 days (PBS + GL-261luc:Fc; n = 6), (FTY720 + GL-261luc:Fc; n = 8), (PBS + GL-261luc:IL12Fc; n = 5), (FTY720 + GL-261luc:IL12Fc; n = 3). (Right) Example of BLI (photon/s) emitted from the ROI around the tumor site.

We therefore concluded that also in the context of IL-12 mediated glioma rejection, priming of tumor-specific T cells involves secondary lymphoid organs.

9.2.2. Tumor-derived IL-12 induces a change in the myeloid infiltration pattern

Given the observation of IL-12 mainly exerting its effects locally, we addressed the question of how tumor-derived IL-12 affects APCs in the tumor microenvironment potentially shaping the anti-tumor immune response.

IL-12 led to a substantial increase of all hematopoietic-derived $CD45^{hi}$ $CD11b^{neg/int/hi}$ cells in the tumor microenvironment (Figure 14A), while the $CD45^{int}$ $CD11b^{+}$ resident microglia population was not affected (Figure 14A, B). We found that absolute numbers of macrophages ($CD64^{+}$ $MertK^{+}$) and moDCs ($CD11c^{+}$ $Ly6C^{low/hi}$ $MHCII^{hi}$ $CD11b^{+}$) were elevated in the presence of IL-12, whereas the frequency of inflammatory monocytes ($Ly6C^{+}$ $MHCII^{-}$) was significantly decreased (Figure 14C-E).

These observations prompted us to study IL-12-mediated glioma suppression in *ccr2*$^{-/-}$ mice. Mice deficient in CCR2 signaling display defective monocyte/macrophage recruitment during immune responses [233] and virtually lack infiltration of $CD45^{hi}$ $CD11b^{hi}$ cells upon glioma induction (Figure 14F). However, the challenge of these mice with GL-261luc:IL12Fc tumor cells conferred a survival advantage equally to WT mice challenged with GL-261luc:IL12Fc tumors (Figure 14G), indicating that CCR2-dependent recruitment of myeloid cells to the tumor site is not required for IL-12-mediated glioma rejection.

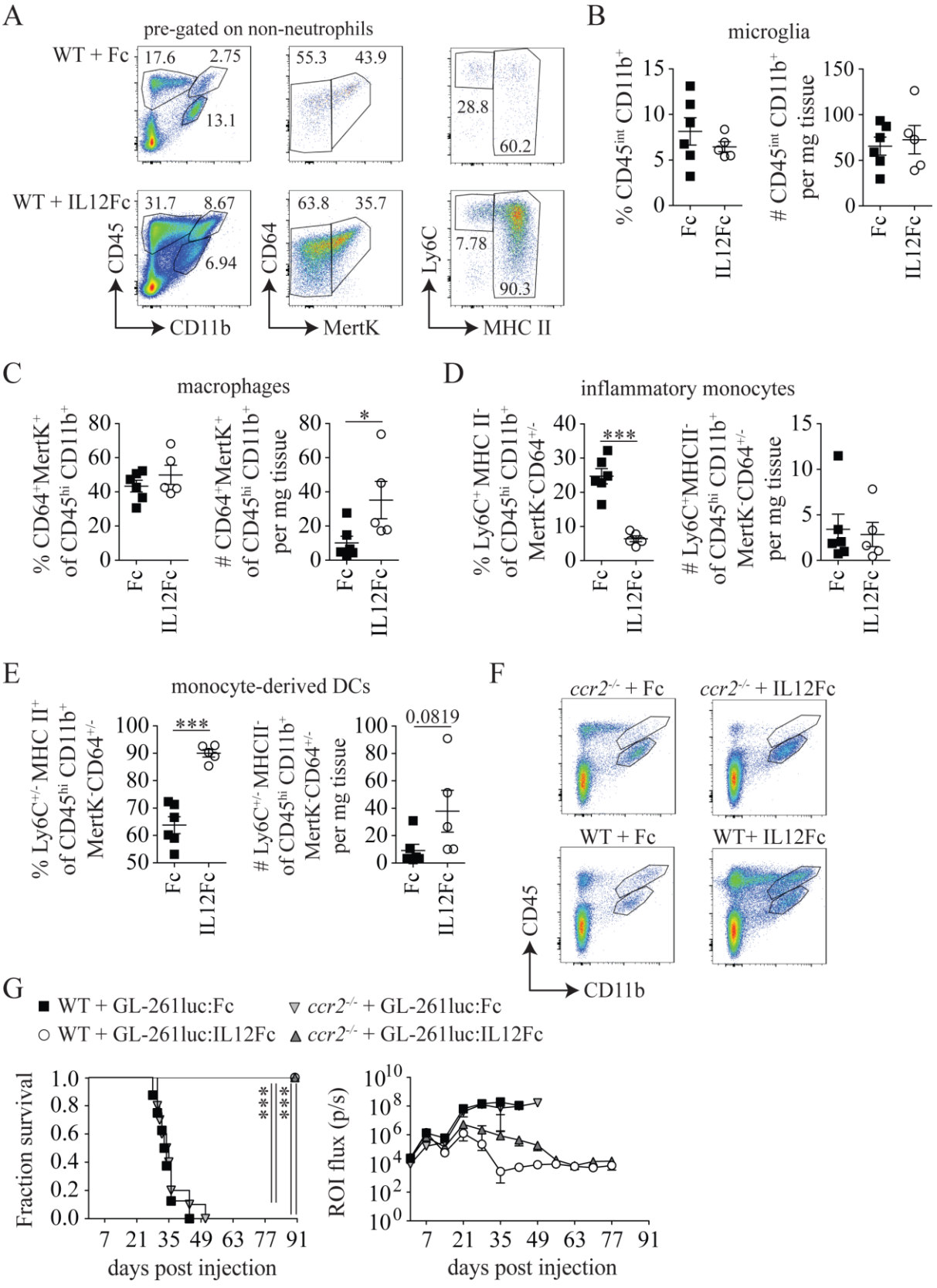

Figure 14: **Local IL-12 release indirectly increases the recruitment CCR2-dependent myeloid cells. (A)** Gating strategy of tumor-infiltrating myeloid cells on day 21 post injection of WT mice bearing GL-261luc:IL12Fc or GL-261luc:Fc tumors pre-gated on non-neutrophils. Macrophages, inflammatory monocytes and moDCs were gated from $CD45^+$ $CD11b^+$ cells, identifying $CD64^+MertK^+$ macrophages and inflammatory monocytes and moDCs within the $MertK^{neg}$ population. Monocytes and moDCs were

distinguished based on MHCII expression **(B-E)** Statistical summary of percentages and absolute numbers (normalized to mg tissue) of $CD45^{int}$ $CD11b^{+}$ microglia, $CD64^{+}$ $MertK^{+}$ macrophages, $Ly6C^{+}$ $MHCII^{-}$ inflammatory monocytes and $MHCII^{+}$ $CD11b^{+}$ $CD11c^{+}$ $MertK^{-}$ monocyte-derived DCs (WT + GL-261luc:Fc; n = 6), (WT + GL-261luc:Fc; n = 5). **(F)** Flow cytometric analysis of infiltrating $CD45^{hi}$ $CD11b^{hi}$ cells in WT and *ccr2*$^{-/-}$ mice challenged with intracranial GL-261luc:IL12Fc or GL-261luc:Fc tumors. **(G)** (Left) Survival of WT or *ccr2*$^{-/-}$ mice challenged with GL-261luc:IL12Fc or GL-261luc:Fc cells monitored over 90 days. (Right) BLI emitted from the ROI around the tumor site measured on a weekly basis (WT + GL-261luc:Fc; n = 8), (WT + GL-261luc:IL12Fc; n = 9), (*ccr2*$^{-/-}$ + GL-261luc:Fc; n = 10), (*ccr2*$^{-/-}$ + GL-261luc:IL12Fc; n = 8). Pooled data from two independent experiments.

Since CCR2-dependent cells were dispensable for tumor rejection, we focused our analysis on other, $CD11b^{-}$ tumor-infiltrating APCs and found that $CD24^{+}CD103^{+}$ migratory DCs were significantly increased in the presence of IL-12 (Figure 15A). We thus decided to study IL-12 tumor rejection in Basic leucine zipper transcription factor ATF-like *(Batf)3*$^{-/-}$ mice. C57BL/6 mice deficient in the transcription factor Batf3 have previously been shown to lack $CD8a^{+}$ DCs only in the spleen while entirely lacking the $CD103^{+}$ migratory DC compartment [234]. Moreover, this population has been shown to be supportive for anti-tumor immune responses in distinct preclinical melanoma models [235-238].

As depicted in Figure 15C, *Batf3*$^{-/-}$ lack $CD103^{+}$ DCs in the tumor microenvironment, even in the presence of IL-12. The challenge of these mice with GL-261luc:IL12Fc tumor cells conferred a survival advantage equally to WT mice challenged with GL-261luc:IL12Fc (Figure 15D). However, following the overall tumor development, mice lacking $CD103^{+}$ DCs had a delay in rejecting IL-12 overexpressing tumors (Figure 15D).

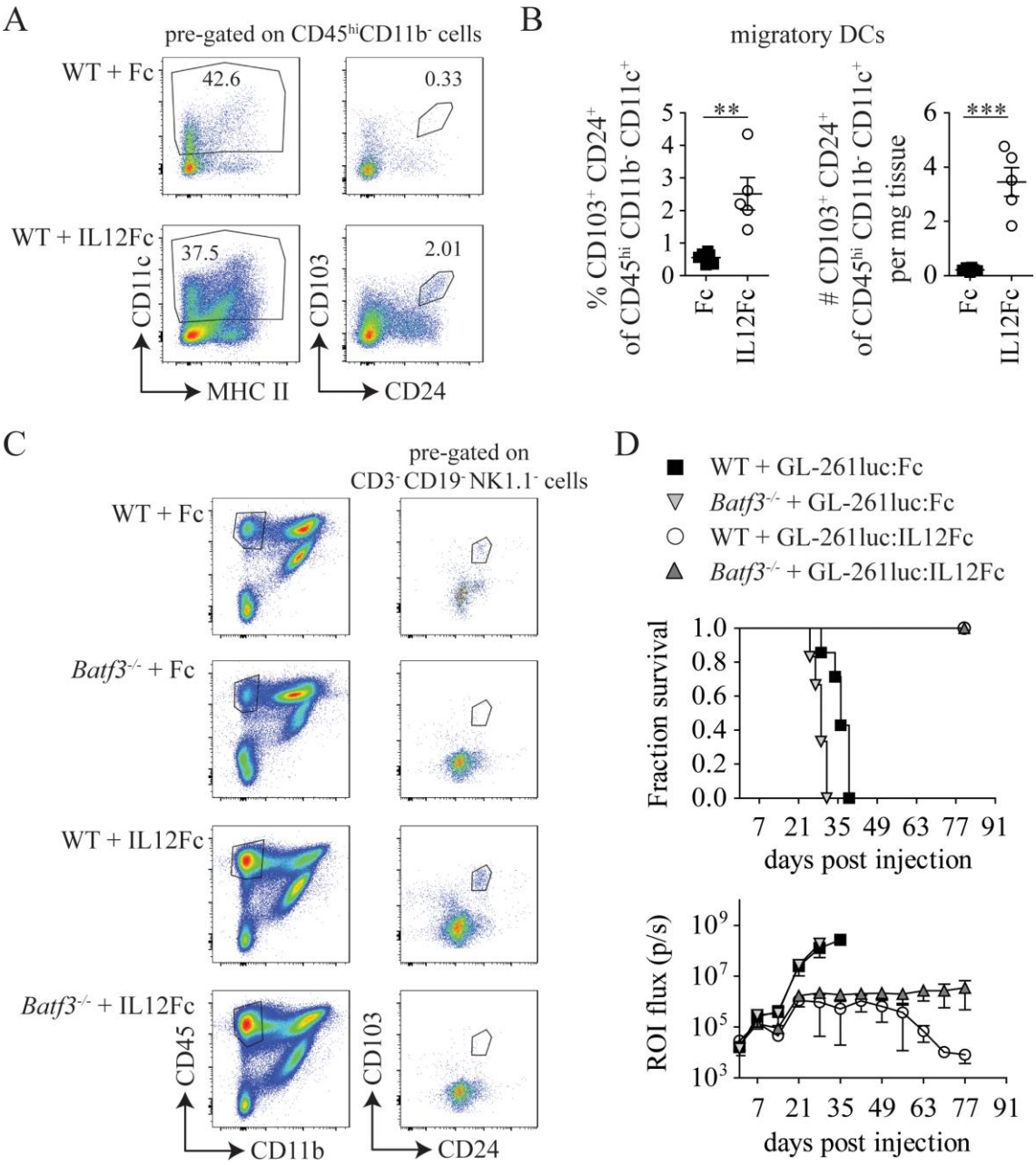

Figure 15: **The role of Batf3 during IL-12 mediated tumor rejection. (A)** Gating strategy for CD103^{+} CD24^{+} CD11c^{+} DCs in the tumor microenvironment pre-gated from CD45^{+} CD11b- cells. **(B)** Statistical summary of percentages and absolute numbers (normalized to mg tissue) of CD103^{+} CD24^{+} CD11c^{+} DCs (WT + GL-261luc:Fc; n = 6), (WT + GL-261luc:Fc; n = 5). **(C)** Representative flow cytometric analysis of infiltrating CD45^{hi}CD11b^{hi} cells and CD103^{+} CD24^{+} DCs in WT and *Batf3*$^{-/-}$ mice challenged with intracranial GL-261luc:IL12Fc or GL-261luc:Fc tumors. **(D)** (Upper) Survival of WT or *Batf3*$^{-/-}$ mice challenged with GL-261luc:IL12Fc or GL-261luc:Fc cells monitored over 90 days. (Lower) BLI emitted from the ROI around the tumor site measured on a weekly basis (WT + GL-261luc:Fc; n = 4), (WT + GL-261luc:IL12Fc; n = 7), (*Batf3*$^{-/-}$ + GL-261luc:Fc; n = 7), (*Batf3*$^{-/-}$ + GL-261luc:IL12Fc; n = 6).

In summary, these results show that lack of myeloid cells due to lack of CCR2 or Batf3 does not impact survival, but lack of Batf3 alters the kinetic of the effector phase.

10. Discussion

Glioblastoma - the most malignant type of brain cancer - is associated with poor prognosis having a 5-year survival rate of 33.6%, despite therapy consisting of maximal surgery, radiotherapy, and chemotherapy [88]. Notably, glioblastoma ultimately relapses in almost all patients, with no treatment option currently available prolonging survival upon relapse [84]. Given the success of immunotherapy with immune checkpoint inhibitors for the treatment of other tumor types, such as the FDA approved checkpoint inhibitors targeting the molecules PD-1 and CTLA-4, immunotherapy has become an appealing treatment strategy for glioblastoma [84, 110-115]. Thus, reverting the suppressive tumor microenvironment into a pro-inflammatory microenvironment to favor tumor control has been a subject of research in multiple preclinical glioma models. Studies primarily focused on strengthening T cell activation either by activating co-stimulatory receptors (e.g., OX-40) or blocking of co-inhibitory receptors (e.g., PD-1 and CTLA-4) combined with radiotherapy or by combining blockade of co-inhibitory receptors with intratumoral cytokine delivery, such as IL-12. Notably, these approaches have been shown to induce anti-tumor responses leading to prolonged survival in distinct preclinical glioma models [84, 105, 116, 117, 120, 207]. In this respect, we have previously shown that reverting the suppressive tumor microenvironment into a pro-inflammatory microenvironment is achievable, by combining intratumoral IL-12 with systemic blockade of the co-inhibitory receptor CTLA-4 on T cells. Moreover, we could show that GL-261 glioma rejection required T cells and was perforin-dependent [120].

While the tumor suppressive capacity of IL-12 has long been known [166, 183, 187, 189], early clinical trials administering IL-12 not only led to low response rates but also to the occurrence of severe adverse side effects resulting in fatalities [166, 223, 239, 240]. Aiming to minimize toxicity while maximizing efficacy, current clinical trials use local administration of IL-12 [209, 216]. In this respect, there has been an increase in registered studies since 2011 on clinicaltrials.gov [208]. However, in addition to targeted delivery to the tumor site, translation into the human situation also requires an immunological understanding of the underlying mechanistics and cell types involved.

10.1. Investigating the IL-12 responsive cell subset

Given the vast amount of studies performed with IL-12 in preclinical models, it has become increasingly clear that IL-12 not only acts tissue-specific but also in a time and dose-dependent fashion. This is best exemplified in studies available in the B16 melanoma model, where overexpression of IL-12 leads to tumor suppression mediated by *Rorγt*-dependent ILCs [185, 186]. In contrast, treatment of established B16 tumors was shown to require adaptive immunity mediated by $CD8^+$ T cells [185-187]. Moreover, when the dose of systemically administered IL-12 was increased, treatment of established B16 tumors involved NK and NKT cell-dependent mechanisms of tumor suppression [183, 188]. To add another level of complexity, established B16 melanoma tumors treated with adoptively transferred $CD8^+$ T cells expressing functional single-chain IL-12 induced a programmatic change in tumor-infiltrating myeloid cells including macrophages, DCs, and MDSCs sensing IL-12. In this experimental setup, myeloid cells sensing IL-12 were crucial for the induction of $CD8^+$ T cell-mediated tumor suppression [241]. In this respect, other studies reported IL-12 receptor expression on cells of myeloid cells origin and tonsillar B cells in addition to the established cell types expressing the IL-12 receptor including ILCs, NKT, and T cells [175, 176]. Moreover, IL-12 receptor expression was found in non-hematopoietic cells, such as keratinocytes involved in mediating a tissue-protective response in the Aldara-induced psoriatic plaque formation model [180]. Regarding preclinical glioma models, studies have suggested both T cells and NK cells to be involved in IL-12 mediated glioma rejection [199-202, 205-207]. Notably, these studies differ in treatment initiation and routes of delivery of intratumoral IL-12. Moreover, the requirement of IL-12 receptor expression on the involved cell types for tumor rejection was not assessed in these studies [120, 203, 207].

Our systematic analyses of the responding cell types required for IL-12 mediated glioma rejection showed that IL-12 tumor rejection relies on BM-derived cells responding to IL-12 (Figure 7). Thus, these findings excluded the involvement of CNS-resident cells, previously reported to mediate recovery in an experimental model of VSV infection of the CNS [177]. Moreover, the requirement of IL-12 receptor-expressing microglia was also discarded, despite the reported upregulation of this receptor in these cells in primary culture conditions upon stimulation with IL-12 [178, 179].

Within the hematopoietic compartment, ILCs, NKT, and T cells represent typical candidate effector populations expressing the IL-12 receptor [120, 166, 183, 185, 187, 189]. However, we previously established that ILCs including NK cells were not required for glioma rejection in our model [120]. Thus, we investigated the involvement of $CD4^+$ and $CD8^+$ T cells regarding IL-12 receptor signaling. While IL-12 receptor signaling in $CD4^+$ T cells was dispensable, we found that depletion of the $CD4^+$ T cell compartment abrogated rejection (Figure 8 and Figure 10). These findings agree with a recent publication of a glioblastoma stem-like cell model (GSC005) treated with a combination of oncolytic virus releasing IL-12 (G47Δ-mIL12) applied together with the blockade of the inhibitory molecules CTLA-4 and PD-1, in which depletion of $CD4^+$ T cells abrogated the efficacy of treatment [207]. In this regard, $CD4^+$ T cells serve multiple purposes, such as providing help for $CD8^+$ T cells and B cells [242]. B cells, require $CD4^+$ T cells for germinal center formation, class switching and affinity maturation. In our experimental setups, IL-12 release by tumor cells induced an influx of B cells (data not shown), however, transfer of merely T cells into *Rag1*$^{-/-}$ mice led to tumor rejection in the absence of B cells. Thus, this rules out the requirement of B cells during IL-12-mediated glioma rejection [175, 243].

Within strong inflammatory settings, $CD8^+$ T cells do not require the support of $CD4^+$ T cells for activation. However, the absence of $CD4^+$ T cells leads to poorly responding memory cells during reactivation [242]. In this regard, we found that functional IL-12 receptor signaling in $CD8^+$ T cells was essential to induce IL-12-driven glioma suppression (Figure 8). The finding of $CD8^+$ T cells required for tumor rejection coincides with our previously published findings showing perforin-dependency during IL-12 mediated tumor rejection [120] and the IL-12-induced enhanced transcription of cytotoxic granule-associated molecules such as perforin and granzyme B [167, 244]. Moreover, tumor-derived IL-12 mainly exerted its effects locally by increasing proliferation of tumor-infiltrating $CD8^+$ T cells (Figure 9). This was shown in an immune competent microenvironment using mixed chimeric mice bearing WT (CD45.1) and *IL12rb2*$^{-/-}$ or WT (CD45.1) and WT (CD45.2) hematopoietic compartments. Also using this experimental setup, no changes were observed in the myeloid compartment (data not shown), ruling out the contribution of these cells sensing IL-12 as described by Kerkar *et al.* in the treatment of B16 melanoma. [241].

Concluding, our results support the mechanism of IL-12-driven glioma rejection to be mainly dependent on the sensing of IL-12 locally by the effector cells, which are then enabled via a stronger "signal 3" to kill proliferating tumor cells more efficiently.

10.2. IL-12 mediated glioma rejection in the context of the glioma-immune cycle

In addition to elucidating the IL-12 responsive cell type required for inducing tumor rejection, we investigated the contribution of IL-12 in generating the anti-tumor immune response within the cancer immune cycle. This multistep process – consisting of antigen release, presentation, priming/activation of T cells in tumor draining lymph nodes, trafficking and infiltration of T cells into tumors, recognition, and killing of cancer cells – has previously been described for malignant tumors of the CNS. In this regard, APCs transporting CNS antigens are known to be drained by the cerebrospinal fluid into cervical lymph nodes [85]. Cervical lymph nodes have been established as the location of priming, leading to clonal expansion, acquisition of effector functions and specific adhesion molecule patterns of tumor-specific T cells [245, 246]. To assess the contribution of IL-12 in priming and shaping the effector response within the glioma immune cycle, we used a tumor model expressing the ovalbumin antigen to enable the tracking of adoptively transferred tumor-specific OT1 $CD8^+$ T cells and OT2 $CD4^+$ T cells (Figure 11 and Figure 12). We found that IL-12 induced CD8 T cell proliferation within the tumor microenvironment, which was limited to pre-activated T cells. This observation coincides with our previous finding showing IL-12 inducing local proliferation of IL-12 receptor expressing polyclonal $CD8^+$ T cells. In contrast, we only detected minor ovalbumin-specific CD8 T cell proliferation in the cervical lymph nodes, an observation likely due to low antigen availability caused by injecting mixtures of tumor cells. Additionally, these observations suggest that the transferred tumor-specific $CD8^+$ T cells in the cervical lymph nodes swiftly egress from the lymph node to the circulation upon activation and subsequently to the tumor site. In this respect, it has previously been published that the final functional maturation of tumor-specific $CD8^+$ T cells in the GL-261 glioma model occurs within the tumor microenvironment [246].

As for the $CD4^+$ T cell compartment, no proliferation was observed, neither in the tumor microenvironment nor the cervical lymph nodes. This suggests that the endogenous tumor-specific $CD4^+$ T cell response is sufficient to sustain tumor-specific $CD8^+$ T cell

immunity and that the availability of MHC class II OVA peptide might be limited in this experimental setup.

Notably, the GL-261 tumor cell line also expresses a wide range of known tumor antigens such as the glycoprotein 100 (gp100) and tyrosine-related protein-2 (Trp-2) [149, 153], increasing its immunogenicity and supporting T cell responses even in the absence of artificially introduced antigens such as ovalbumin. While in mice, many cancer immunotherapeutics showed robust efficacy, similar results have been difficult to achieve in humans. This discrepancy might be due to the neo-epitope load in murine cell lines which might not reflect the human situation that usually shows a variable but rather low amount of mutations. Even though human glioblastoma carries an intermediate mutational load compared to other tumor types [247, 248], the necessity of improving antigen presentation in glioblastoma treatment is exemplified by multiple strategies currently being exploited. Among those approaches are loading of glioblastoma-associated antigens/glioblastoma specific antigens and manipulation of co-stimulatory and co-inhibitory signals of DCs to boost anti-tumor immunity [249].

Nevertheless, not observing any proliferation of ovalbumin-specific T cells in the cervical lymph nodes prompted us to study the role of peripheral versus *in situ* priming during IL-12 mediated glioma rejection. The blockade of lymphocytes within secondary lymphoid organs by Fingolimod showed that IL-12 mediated glioma rejection does require peripheral priming (Figure 13). These results are in line with our previous findings of CCR7 and lymphotoxin-beta receptor (Ltbr) dependency during IL-12 glioma challenge (data not shown). Given that IL-12 mediated tumor rejection required peripheral priming, even though we could not detect proliferating tumor-specific T cells in the cervical lymph nodes, we focused on the interaction of myeloid cells and T cells within the tumor microenvironment.

Our thorough characterization of the tumor microenvironment revealed that tumor-derived IL-12 led to a substantial infiltration of all myeloid cells into the tumor microenvironment (Figure 14). Coinciding with previous reports, we found that moDCs displayed the main fraction of infiltrating myeloid cells in the context of local IL-12 release in the GL-261 tumor model [250]. Despite the functional role that moDCs have been given in establishing robust T cell responses leading to tumor clearance, we found that moDCs and macrophages were dispensable for IL-12 mediated tumor rejection in our

model. These results contradict a recently published study showing a key role of F4/80$^+$ macrophages in glioblastoma rejection upon CTLA-4, PD-1 and IL-12 treatment [207]. The differences in the glioblastoma models used and the combination with checkpoint blockade could, however, explain a differential requirement of cell types for tumor rejection.

We could not observe an increase of microglia in the presence of IL-12 compared to control tumors. Even though microglia cells can cross-present tumor antigens both *in vitro* and *in vivo* [251, 252], they are known to inefficiently present glioma antigens to cytotoxic CD8$^+$ T cells [253]. Also, they have been shown to support immunosuppression through the release of cytokines such as TGF-β and IL-10 and by losing MHC expression [77, 254]. To fully understand the contribution of microglia during IL-12 mediated glioma rejection would require the generation of BM chimeras using MHCI$^{-/-}$ and MHCII$^{-/-}$ into WT (and vice versa). Alternatively, co-culture assays using sorted microglia from ovalbumin-expressing tumors and infiltrating DCs cultured with tumor-specific OT1 CD8$^+$ or OT2 CD4$^+$ T cells would need to be performed. However, the low number of DC infiltrates, and microglia found in GL-261 tumors provide a significant hurdle to perform assays of such kind.

By looking beyond CCR2-dependent cells and the response of resident microglia, we found that IL-12 increased the expansion of a small subset of CD103$^+$ DCs. This population has previously been shown to be supportive for anti-tumor immune responses in distinct melanoma models [235-238]. The development of CD103$^+$ DCs is dependent on the transcription factors interferon regulatory factor 8 (IRF8), zinc finger and BTB domain containing 46 (Zbtb46) and Batf3. Moreover, CD103$^+$ DCs are generated upon stimulation with GM-CSF and Fms-like tyrosine kinase-3 ligand (Flt3L). Notably, CD103$^+$ DCs have been found to be increased in regressing tumors in both humans and mice [235]. Migrating in a CCR7-dependent fashion, this type of DC has been shown to support T cell-mediated tumor suppression and to be required for expansion and activation at the tumor site enhancing the response to combined PD-L1 and BRAF inhibition [236, 237]. Moreover, Spranger *et al.* showed that effector T cell trafficking and adoptive T cell therapy rely on CD103$^+$ DCs found in inflamed tumors [238]. Our data shows that *Batf3*$^{-/-}$ mice lacking CD103$^+$ migratory DCs succumbed to glioma slightly earlier compared to WT controls in the absence of IL-12, suggesting a supportive role of Batf3-dependent DCs for the activation of T cells. However, IL-12 conferred

survival in *Batf3*$^{-/-}$ mice as it did with the WT counterparts (Figure 15). Notably, tumor growth is slightly different with WT mice being able to reject, while *Batf3*$^{-/-}$ mice seem to control the tumors, but are not able to reject IL-12 expressing tumors. These results suggest that CD103^{+} DCs support the effector phase of T cell-mediated tumor killing, but also that other cellular players are involved in T cell priming and can partially compensate for the lack of migratory DCs. One hypothesis to the fact that mice still survive in the absence of CD103^{+} DCs could be due to the capacity of GL-261 tumor cells to upregulate MHC expression in response to IFN-γ [149]. Even though we previously established that IL-12 mediated glioma rejection is independent of IFN-γ, it is conceivable that IFN-γ contributes to the compensatory mechanism leading to tumor cell killing. In this respect, it would be interesting to determine the extent of MHC upregulation by flow cytometry on *ex vivo* tumor cells in the context of IL-12.

10.3. Concluding remarks and future directions

Using a preclinical glioma model, we elucidated the mechanism of IL-12 mediated tumor rejection. Mainly exerting its effects on tumor-infiltrating cytotoxic CD8^{+} T cells, we found that IL-12 induces an infiltration of myeloid cells into the tumor microenvironment, amongst which migratory DCs supported the effector phase of rejection. Since mice lacking migratory DCs tolerated, albeit struggled to reject, their tumors, we propose that the direct interaction of tumor cells with cytotoxic CD8^{+} T cells is critical for the killing of tumor cells. In this respect, from a translational perspective, it would be interesting to investigate ways to modulate influx and function of CD103^{+} DCs into tumors. Given the intermediate mutational burden in glioblastoma, it would be interesting to exploit distinct strategies aiming to enhance DC efficacy as an additional treatment angle, improving the immune response even in less immunogenic settings than the GL-261 tumor model. While early treatment with Flt3L, driving DC development, led to inhibition of glioma progression [255-257], it would be interesting to determine whether late-stage therapy with Flt3L in combination with IL-12 or anti-CTLA-4 blockade can confer survival.

Given that no good predictive biomarker is currently available to assess treatment efficacy, it would also be interesting to investigate CD103^{+} DCs as a potential biomarker for the response pre/post therapy of responders vs. non-responders.

Concluding, our findings provide new insights into the mechanism of IL-12 mediated glioma rejection. These include the requirement of directly and indirectly responsive cell types to consider as targets to boost anti-tumor immunity.

12. References

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14. Appendix

Disclaimer

The appendix is based on the following manuscript:

"Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell–mediated glioma rejection."

Johannes vom Berg, Melissa Vrohlings, Sergio Haller, Aladin Haimovici, Paulina Kulig, Anna Sledzinska, Michael Weller, and Burkhard Becher.

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Contribution: M.V. contributed the data on the CTLA-4 expression on TILs (Figure 2F) comparing Fc and IL-12Fc tumors. Moreover, M.V. performed the experiment of *perforin-1* gene expression by NK, $CD4^+$ and $CD8^+$ T cells infiltrating the tumor (Figure 4E).

JEM

Brief Definitive Report

Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell–mediated glioma rejection

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Glioblastomas (GBs) are the most aggressive form of primary brain cancer and virtually incurable. Accumulation of regulatory T (T reg) cells in GBs is thought to contribute to the dampening of antitumor immunity. Using a syngeneic mouse model for GB, we tested whether local delivery of cytokines could render the immunosuppressive GB microenvironment conducive to an antitumor immune response. IL-12 but not IL-23 reversed GB-induced immunosuppression and led to tumor clearance. In contrast to models of skin or lung cancer, IL-12–mediated glioma rejection was T cell dependent and elicited potent immunological memory. To translate these findings into a clinically relevant setting, we allowed for GB progression before initiating therapy. Combined intratumoral IL-12 application with systemic blockade of the co-inhibitory receptor CTLA-4 on T cells led to tumor eradication even at advanced disease stages where monotherapy with either IL-12 or CTLA-4 blockade failed. The combination of IL-12 and CTLA-4 blockade acts predominantly on CD4$^+$ cells, causing a drastic decrease in FoxP3$^+$ T reg cells and an increase in effector T (T eff) cells. Our data provide compelling preclinical findings warranting swift translation into clinical trials in GB and represent a promising approach to increase response rates of CTLA-4 blockade in solid tumors.

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Abbreviations used: ADCC, antibody-dependent cell-mediated cytotoxicity; BLI, bioluminescence imaging; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; GB, glioblastoma; HPRT, hypoxanthine-guanine phosphoribosyltransferase; ILC, innate lymphoid cells; i.t., intratumoral; MFI, mean fluorescent intensity; NKT, NK T cells; *Prf1*, *Perforin*; ROI, region of interest; T eff, effector T; TIL, tumor-infiltrating lymphocyte; T reg, regulatory T.

Glioblastoma (GB) is among the most aggressive cancers known. Current treatment options are limited and the clinical prognosis is poor. Patients diagnosed with GB show a median survival of little more than a year despite aggressive surgery, radiation therapy, and chemotherapy (Weller et al., 2013). Moreover, GBs induce a highly immunosuppressive microenvironment, characterized by the presence of T reg cells (Grauer et al., 2007; Jacobs et al., 2010). Given the failure of conventional therapy in GBs, the most promising treatment option may thus rely on the exploration of immunotherapeutic strategies. IL-12 is the prototype member of a group of heterodimeric cytokines with predominantly proinflammatory properties. IL-12 polarizes naive helper T cells (T_H) to adopt a T_H1 phenotype and stimulates cytotoxic T cells, NK T (NKT) cells, and conventional NK cells. The therapeutic success of application of IL-12 in various preclinical animal models of cancer is compelling (Colombo and Trinchieri, 2002). However, in humans, systemic delivery of IL-12 evoked serious adverse events such as leukopenia and thrombocytopenia, including fatalities in two patients, at moderately effective doses (Atkins et al., 1997; Leonard et al., 1997). Thus, local rather than systemic delivery of IL-12 represents the only viable option for using IL-12 in cancer immunotherapy in humans. IL-12 appears to exert its cancer-suppressive properties through different effector cells in a tissue-specific manner. In the B16 melanoma model, IL-12–mediated suppression of s.c. tumor growth is mediated by a small

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population of IL-12–responsive, Rorγt-dependent innate lymphoid cells (ILCs; Eisenring et al., 2010). On the other hand, B16-derived lung tumors are controlled through IL-12–activated NK cells (Kodama et al., 1999; Eisenring et al., 2010). Conversely, IL-12–mediated glioma control has been attributed to T cells and NK cells, but open questions remain about which cell types indeed are the precise cellular targets of IL-12, consequently mediating anti-GB immunity (Vetter et al., 2009; Yamanaka et al., 2002, 2003). IL-23 is another member of the IL-12 family and also has potent pro-inflammatory properties. Several groups reported potent antitumor activity in various experimental settings including brain tumors (Lo et al., 2003; Hu et al., 2006). Others have reported a protumorigenic effect of IL-23 (Langowski et al., 2006). The goal of this study was to systematically analyze whether and how IL-12 and IL-23 induce an antitumor immune response in a syngeneic murine model of GB.

RESULTS AND DISCUSSION

To determine whether IL-12 and IL-23 are suitable candidates to overcome the local immunosuppressive environment in GB and to trigger rejection, we expressed either of these two cytokines in C57BL/6 syngeneic GL-261 mouse glioma cells (Szatmári et al., 2006). First, we generated a GL-261 line that constitutively expressed *Photinus pyralis* luciferase (hereafter, termed GL-261luc) for bioluminescence imaging (BLI). We next modified this cell line to continuously release a fusion protein of IL-12 or IL-23 joined to the crystallizable fragment of mouse IgG3 (IL-12Fc or IL-23Fc) or the IgG fragment alone as control (termed GL-261luc:IL-12, GL-261luc:IL-23, and GL-261luc:Fc, respectively). Cytokine production and BLI were equivalent among transfected cells (unpublished data). Expression levels of MHCI and II and proliferation were comparable to parental cells (Fig. 1, A and B), as was the median survival of animals inoculated with GL-261luc:Fc (Fig. 1 C).

We implanted GL-261luc:IL-12 or GL-261luc:IL-23 intracranially into C57BL/6 mice and compared growth with GL-261luc:Fc, monitoring BLI and survival (Fig. 1, D and E). While IL-23 secretion appeared to be mildly tumor promoting (Fig. 1 D), IL-12 expression conferred a clear survival advantage (Fig. 1 E). This was not a tumor intrinsic effect, as GL-261luc:Il-12 displayed progressive growth in *Il12rb2*$^{-/-}$ hosts (unpublished data). On day 35 after injection, we detected only a residual tumor in some animals injected with GL-261luc:IL-12, whereas mice challenged with GL-261luc:IL-23 or GL-261luc:Fc showed robust tumor formation when analyzed histologically (Fig. 1 G). This contrasts studies on IL-23–induced glioma rejection using neural stemlike cell– or DC-based approaches that showed potent antiglioma activity (Hu et al., 2006; Yuan et al., 2006). However, in various other models of solid tumors, it is also becoming increasingly apparent that IL-23 has primarily protumorigenic effects (Ngiow et al., 2013). Therefore, for the remainder of this study, we focused on the effector mechanisms of IL-12–mediated glioma rejection.

Longitudinal analysis via histology and flow cytometry of the tumor-infiltrating lymphocyte (TIL) composition revealed that T cells and NK cells were readily detected within tumors 2 wk after tumor implantation (unpublished data). To systematically examine the contribution of specific leukocyte populations, we challenged a series of mouse mutants with intracranial GL-261luc:IL-12. We used mice lacking T and B cells (*Rag1*$^{-/-}$), conventional NK cells (*Il15ra*$^{-/-}$), or mice lacking T, B, NK cells, as well as ILCs (*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$; Fig. 2 A). Tumor protection was lost in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ and *Rag1*$^{-/-}$ animals, whereas *Il15ra*$^{-/-}$ mice were able to control the tumor, suggesting that adaptive lymphocytes are absolutely required for IL-12–mediated glioma rejection. The fact that the loss of NK cells and ILCs in *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice did not change the clinical course compared with *Rag1*$^{-/-}$ mice, combined with the ability of *Il15ra*$^{-/-}$ mice to reject GL-261luc:IL-12, demonstrates that NK cells and ILCs were largely dispensable for IL-12–mediated tumor rejection. We also investigated the contribution of CD4 and CD8 T cells using MHCII (*Ia(b)*$^{-/-}$)- and MHCI (β*2m*$^{-/-}$)-deficient mice, respectively. In contrast to WT mice, *Ia(b)*$^{-/-}$ mice did not control GL-261luc:IL-12 tumors, and β*2m*$^{-/-}$ mice succumbed to the gliomas shortly thereafter (Fig. 2 B). Although there is precedence for a role of NKT cells in IL-12–mediated tumor rejection (Cui et al., 1997), the effective IL-12–mediated tumor rejection in *Il15ra*$^{-/-}$ mice further dismisses a critical role of NK and NKT cells because both populations depend on IL-15R signaling (Gordy et al., 2011).

To further characterize the T cell–dependent tumor control, we tested for immunological memory formation in the surviving mice that had been previously challenged with GL-261luc:IL-12 cells (Fig. 2 C). In contrast to the primary challenge, we now injected GL-261luc:Fc cells into the contralateral hemisphere of survivors or naive WT animals. In agreement with earlier studies (Daga et al., 2007; Liu et al., 2002), we observed a rapid rejection of the newly implanted tumors within days in rechallenged survivors. Whereas BLI at day 1 after injection suggested identical tumor cell seeding across the two groups, only the naive mice exhibited a steadily increasing signal (Fig. 2 C), suggesting memory formation.

IL-12 is recognized for its capacity to polarize IFN-γ–producing T_H1 cells. However, *Ifng*$^{-/-}$ mice rejected GL-261luc:IL-12 cells (Fig. 2 D) at a similar rate as WT animals, suggesting that the mechanism by which IL-12 induces tumor rejection is largely independent of IFN-γ. Conversely, IL-12 also stimulates the cytotoxic activity of T cells. We analyzed the role of perforin, a cytolytic molecule primarily expressed by CD8$^+$ CTLs and NK cells. In contrast to *Ifng*$^{-/-}$ mice, *Prf1*$^{-/-}$ mice failed to control the tumor (Fig. 2 E). This further shows that perforin-mediated T cell cytotoxicity is the major effector mechanism of IL-12–mediated glioma rejection.

Previous studies investigated the mechanisms of IL-12 in glioma rejection, many of these in a DC vaccination setting (Joki et al., 1999; Yamanaka et al., 2002, 2003). A crucial involvement of NK cells and T cells (CD4$^+$ and/or CD8$^+$) in the IL-12–mediated rejection of experimental gliomas was described (Joki et al., 1999; Yamanaka et al., 2002, 2003). These reports present contradictory findings regarding the contribution

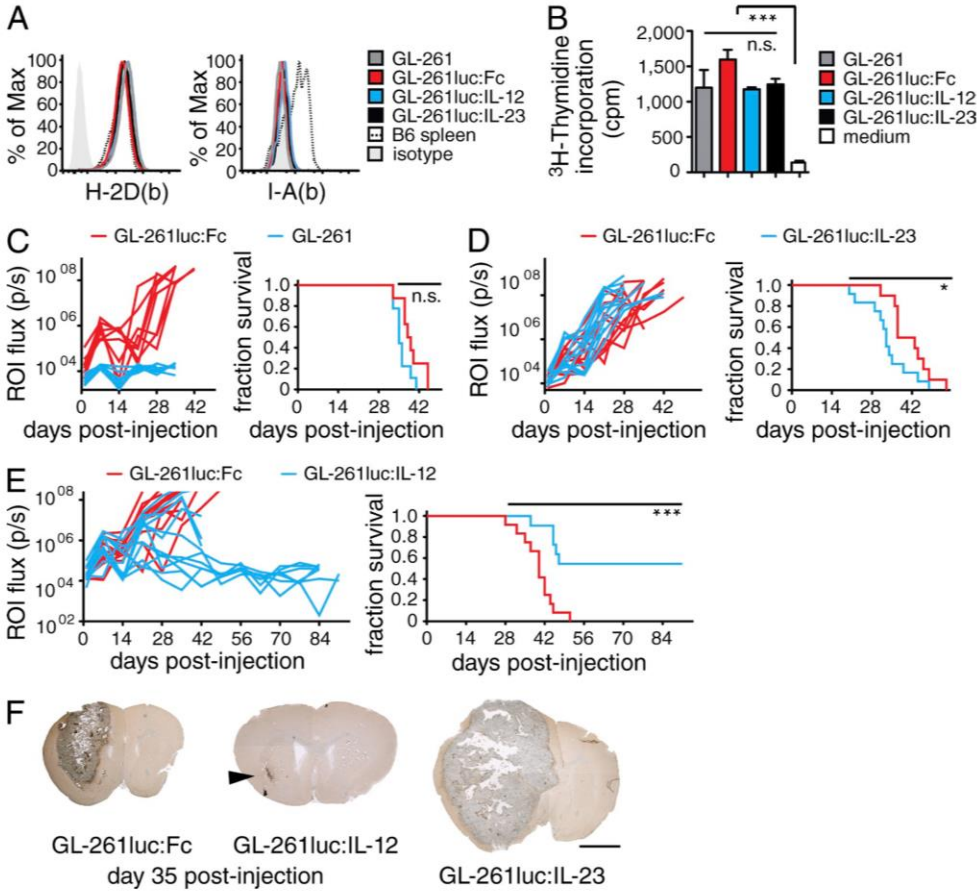

Figure 1. Intratumoral expression of IL-12 but not IL-23 leads to rejection of experimental gliomas. (A and B) In vitro analysis of GL-261, GL-261luc:Fc, GL-261luc:IL-12 and GL-261luc:IL-23 cells; data representative of ≥3 independent experiments: (A) MHCI (H-2D(b)) and MHCII (I-A(b)) expression. (B) ^{3}[H]-Thymidine incorporation of GL-261 cells/well, error bars represent SD, one-way ANOVA with Bonferroni post-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C–E) GL-261 cells were injected into the right striatum of WT animals. (left) BLI emitted from a circular region of interest (ROI) around the tumor site, each trace represents BLI of an individual animal; (right) survival plot of the same experiment, two independent experiments pooled; Log-Rank test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Survival of mice challenged with GL-261 or GL-261luc:Fc ($n \geq 8$/group), with GL-261luc:Fc or GL-261luc:IL-23 cells (D; $n \geq 10$/group, $P = 0.0432$) or GL-261luc:Fc or GL-261luc:IL-12 cells (E; $n = 12$/group, $P = 0.0002$). (F) Largest cross-section of representative tumors ($n = 6$/group) at day 35, brown: F4/80 immunoreactivity; counterstained with hematoxylin, arrow head: GL-261luc:IL-12 tumor. Bar, 2 mm.

of NK cells and the T_H1 hallmark cytokine IFN-γ compared with a study where IL-12 was produced in situ and mouse mutants were used (Vetter et al., 2009). Some of these studies have investigated tumors derived from s.c. injection of glioma cell lines (Joki et al., 1999). However, in s.c. tumors, a subpopulation of ILCs seems to be crucial for IL-12–mediated tumor control, regardless of the tissue origin of the tumor cells used (Eisenring et al., 2010). Using an IL-12–expressing syngeneic glioma cell line and various genetic mutants, we established T cells as the crucial effector cell type of IL-12–mediated glioma rejection. Further characterization of the tumor-infiltrating T cells revealed robust expression of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4; Fig. 2 F).

CTLA-4 is the prototypic co-inhibitory receptor limiting T cell responses and its blockade has been shown to boost antitumor activity in metastatic melanoma in patients (Hodi et al., 2010; Walker and Sansom, 2011). We observed a slight increase in the numbers of CTLA-4–positive $CD4^{+}FoxP3^{-}$ T cells in GL-261luc:IL-12 tumors and an increase in the expression levels of CTLA-4 in T reg cells (Fig. 2 G).

Next, we explored the possibility of combining intratumoral (i.t.) IL-12 therapy with blockade of cytotoxic CTLA-4. For this, we expressed and purified IL-12Fc, which has biological activity that is identical to heterodimeric IL-12 (Fig. 3 A; Eisenring et al., 2010). Switching to a therapeutic setting, we challenged mice with GL-261luc:Fc, and then allowed for

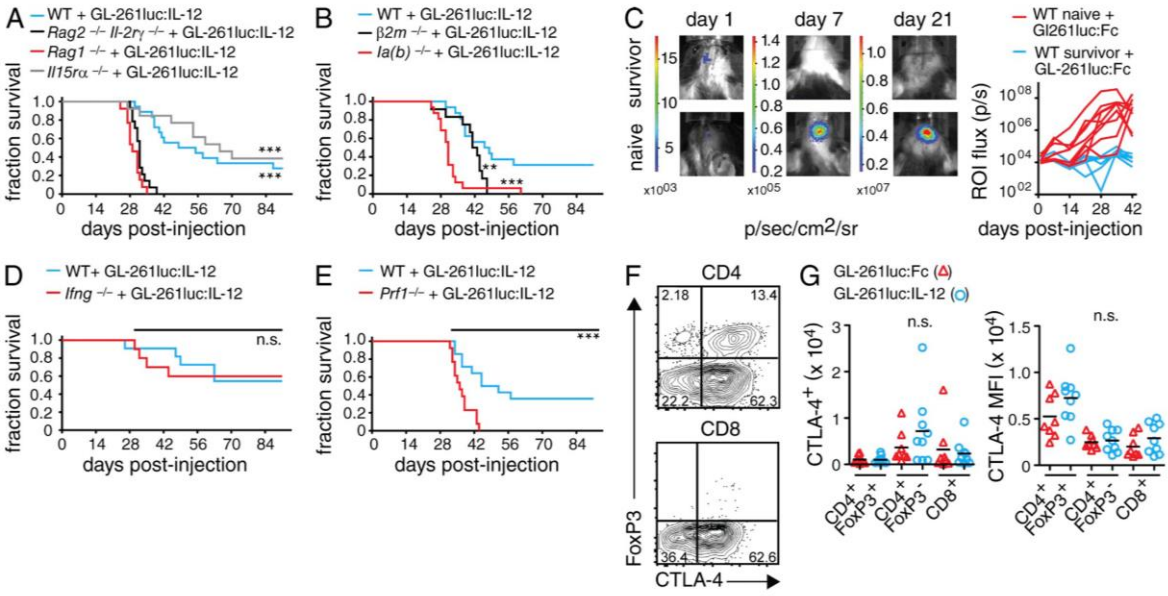

Figure 2. T cell dependency of IL-12–mediated glioma rejection. (A, B and D, E) GL-261luc:IL-12 cells were implanted i.c. into various immunodeficient mouse strains. Animals were monitored for up to 91 d; Log-Rank test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (A) Survival of WT, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, *Rag1*$^{-/-}$, and *Il15ra*$^{-/-}$ mice ($n \geq 13$/group); p(*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ vs. WT), p(*Rag1*$^{-/-}$ vs. WT), p(*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ vs. *Il15ra*$^{-/-}$) and p(*Rag1*$^{-/-}$ vs. *Il15ra*$^{-/-}$) < 0.0001. (B) Survival of WT, β*2m*$^{-/-}$ and *Ia(b)*$^{-/-}$ mice ($n \geq 12$/group); p(β*2m*$^{-/-}$ vs. WT) = 0.0052, p(*Ia(b)*$^{-/-}$ vs. WT) < 0.0001. (C) Rechallenge of naive WT animals and survivors (that had been previously challenged with GL-261luc:IL-12 in the contralateral hemisphere) with GL-261luc:Fc cells; (left) representative BLI at days 1, 7, and 21; (right) BLI quantification ($n \geq 7$/group). (D) Survival of WT and *Ifng*$^{-/-}$ mice ($n \geq 10$/group) injected with GL-261luc:IL-12 (E) Survival of WT and *Prf1*$^{-/-}$ mice ($n \geq 13$/group); p(*Prf1*$^{-/-}$ vs. WT) = 0.0005. (F and G) WT mice were implanted i.c. with GL-261luc:Fc or GL-261luc:IL-12 and analyzed for TILs at day 21 using flow cytometry. (F) Representative contour plot (GL-261luc:Fc TILs gated on live CD45^{hi}/CD11b^{-}/NK1.1^{-} cells). (G) Number (left) of CTLA-4 positive cells and CTLA-4 mean fluorescent intensity (MFI, right) for live CD4^{+}FoxP3^{+}, CD4^{+}FoxP3^{-}, or CD8^{+} T cells ($n \geq 8$/group). One-way ANOVA with Bonferroni post-test; pooled data from two to three independent experiments each; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tumor growth until day 21. At this time point, the cerebral tumor was clearly visible and comparable among the groups as quantified by BLI (Fig. 3, B and C). We implanted osmotic minipumps to deliver purified IL-12Fc over a 28-d period directly into the bulk tumor. Local IL-12Fc treatment was combined with systemic administration of antibodies against CTLA-4. Monotherapies with i.t. application of IL-12Fc or systemic anti–CTLA-4 alone conferred only a minor or no survival advantage, respectively (Fig. 3 B). Strikingly, local IL-12Fc administration directly into the tumor site in combination with systemic CTLA-4 blockade led to a full remission in most mice. We observed similar survival rates treating GL-261luc:Fc- or GL-261-derived tumors with this combination therapy (unpublished data), indicating its effectiveness independent of luciferase as xeno/neo antigen.

2 wk after the start of treatment (day 35 after injection), signs of a successful antitumor immune response were visible for IL-12Fc/PBS and IL-12Fc/αCTLA-4 treated animals in histological overviews (Fig. 3 C). Further analysis revealed a dramatic increase in CD4^{+} T cells upon IL-12Fc treatment, whereas CD8^{+} T cells increased, especially upon concomitant anti–CTLA-4 treatment (Fig. 3, D and E). In contrast to T cells, NK cells were present in high numbers in PBS/PBS- or PBS/αCTLA-4–treated tumors, but decreased considerably upon IL-12Fc treatment (Fig. 3, D and E). This observation was confirmed by flow cytometry (Fig. 4 A). The drop in NK cells suggests that initially reacting NK cells may subsequently be replaced by T eff cells.

FACS quantification further revealed an increase in total CD4^{+} T cells between days 21 and 35 after injection (Fig. 4 A). Most importantly, there was a strong increase in the frequency of CD4^{+} T cells producing IFN-γ, but a profound reduction of FoxP3^{+} cells (Fig. 4 A). This was also reflected in the ratios of CD8^{+} per CD4^{+}FoxP3^{+} cells and especially CD4^{+}IFN-γ^{+} per CD4^{+}FoxP3^{+} cells, revealing a significant difference between IL-12Fc monotherapy and IL-12Fc/αCTLA-4 combination therapy (Fig. 4 B). The fact that accumulation of T reg cells is a key feature of human GB and correlates with outcome (Jacobs et al., 2010) underlines the clinical significance of the treatment approach used here. During and after treatment, we did not observe any overt symptoms of autoimmunity such as weight loss or paralysis (data not shown). Still, 91 d after tumor inoculation, histological assessment of the brain tissue of surviving animals did show residual infiltrates consisting mainly of CD4^{+} T cells and NK cells (Fig. 3, C and D).

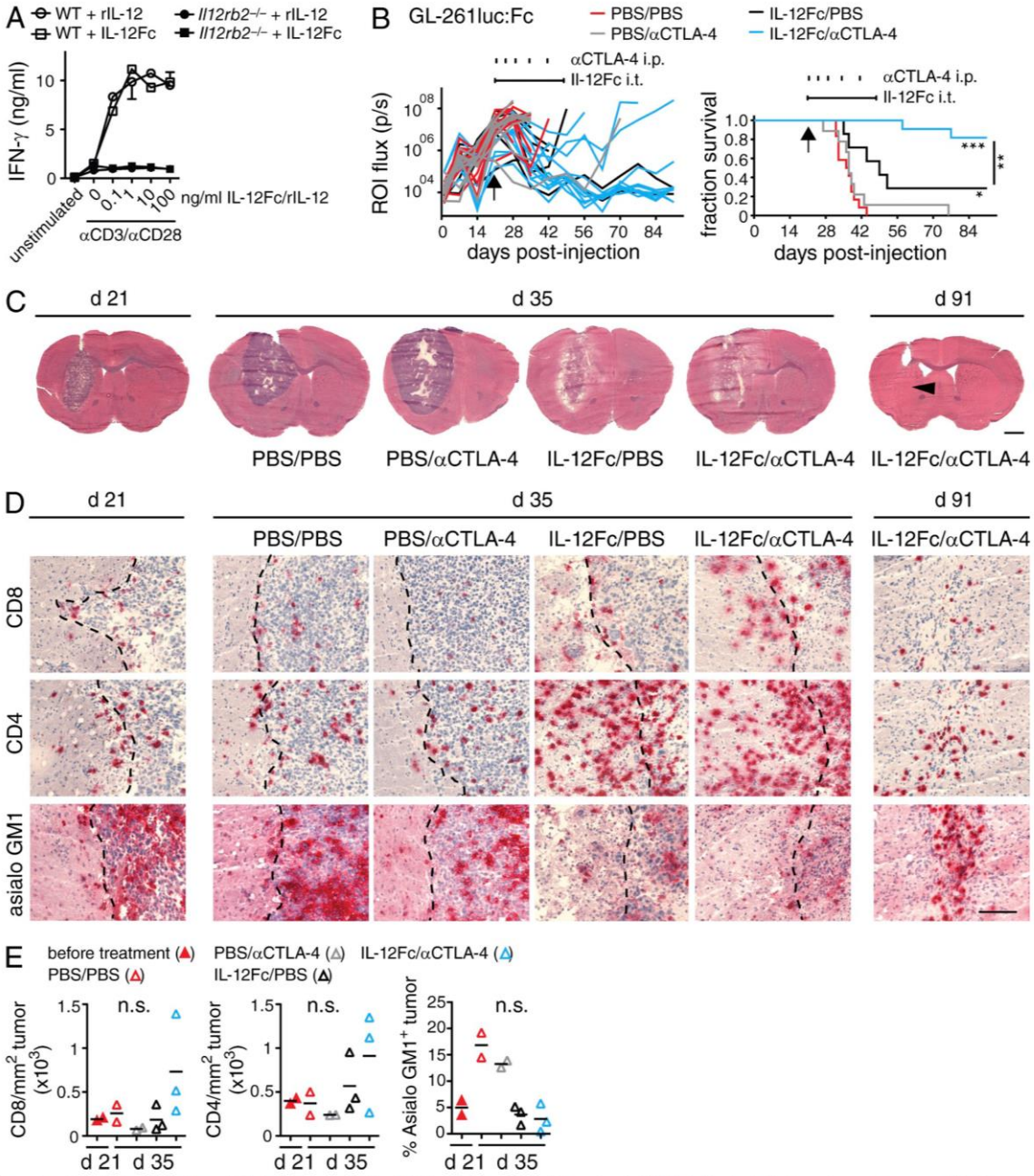

Figure 3. Local administration of IL-12Fc in established GB in combination with systemic CTLA-4 blockade induces tumor clearance. (A) IFN-γ production of WT or *Il12rb2*$^{-/-}$ splenocytes stimulated with anti-CD3 and anti-CD28 mAbs and increasing amounts of heterodimeric IL-12 (rIL-12) or IL-12Fc; one out of three experiments shown. (B) WT mice were injected i.c. with GL-261luc:Fc cells. On day 21 after injection, osmotic minipumps delivering IL-12Fc or PBS intratumorally (i.t.) were implanted as indicated by a horizontal line above graphs. In addition, animals received i.p. injections with αCTLA-4 mAbs or PBS, indicated by vertical tick marks; $n = 7–12$/group; three independent experiments pooled. Log-rank test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; p(IL-12Fc/PBS vs. IL-12Fc/αCTLA-4) = 0.0061, p(PBS/PBS vs. IL-12Fc/PBS) = 0.0160, p(PBS/PBS vs. IL-12Fc/αCTLA-4), and p(PBS/αCTLA-4 vs. IL-12Fc/αCTLA-4) < 0.0001. (C–E) Histological analysis of GL-261luc:Fc tumors at day 21, 2 wk after initiation of treatment (day 35) and at the end of the experiment (day 91). (C) Overviews, arrowhead indicates former tumor center. Bar, 2 mm. (D) Higher magnification, sections show CD4, CD8, and asialo GM1 immunoreactivity (red); tumor margin is indicated (dotted line. Bar, 200 µm. (E) Quantification of TILs/tumor area on histological sections, each dot represents mean of a single animal; one-way ANOVA with Bonferroni post-test; one of two independent experiments shown; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

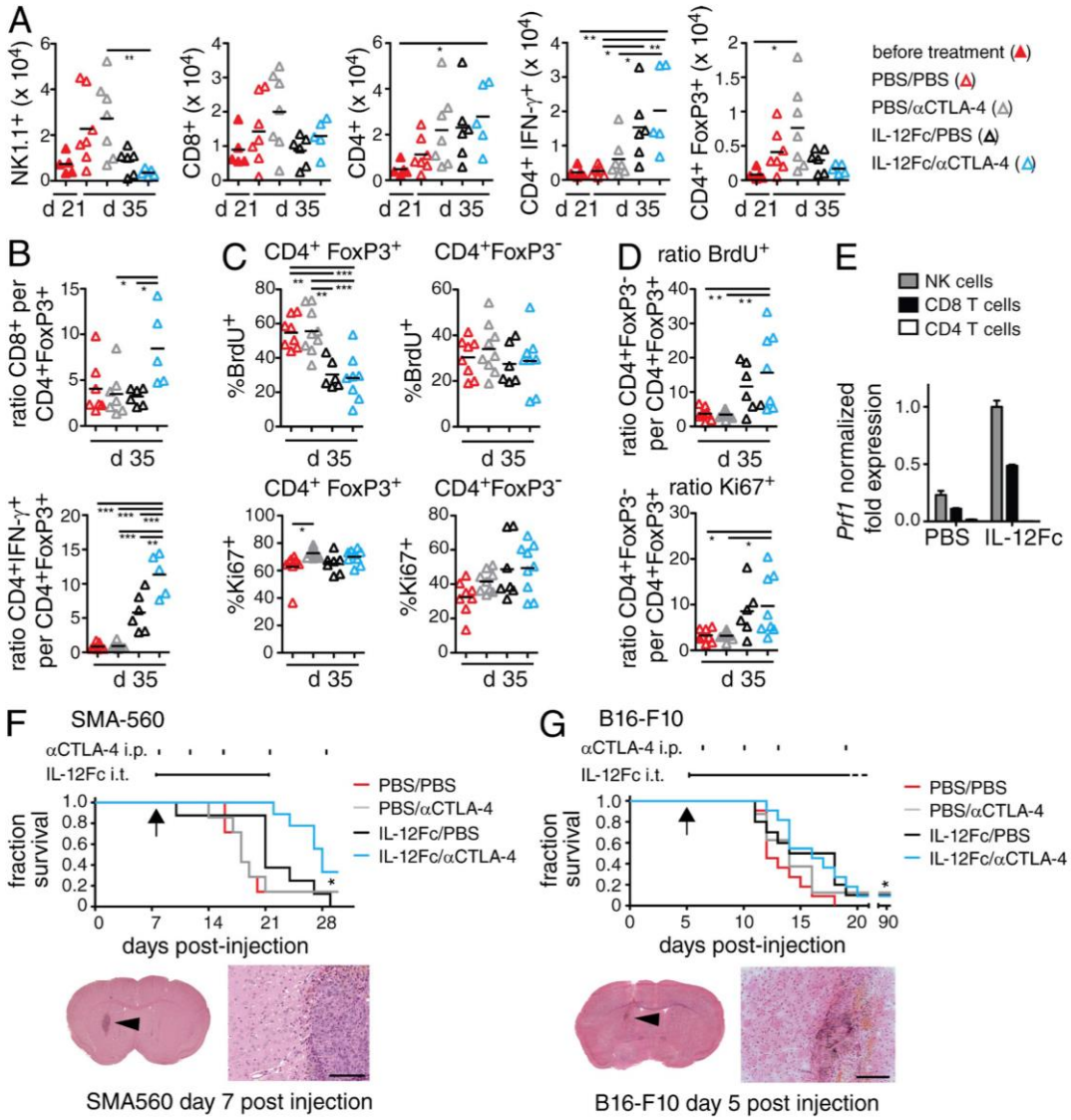

Figure 4. Combination treatment leads to a shift from T reg to T eff cells within the tumor. (A–D) TILs of tumor-bearing brains were analyzed by flow cytometry at days 21 and 35, 2 wk after initiation of treatment; two independent experiments pooled, one-way ANOVA with Bonferroni post-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (A) Total number of NK and T cells, pregated on $CD45^{hi}/CD11b^{-}$ cells. (B) Ratio of $CD8^{+}$ and $CD4^{+}IFN\text{-}\gamma^{+}$ T cells per $CD4^{+}FoxP3^{+}$ T cells; $n \geq 5$/group; (C) BrdU and Ki67 labeling of T reg and T eff cells (pregated on $CD45^{+}CD11b^{-}NK1.1^{-}$) in percent positive cells and (D) ratio of total numbers of T reg versus T eff cells; $n \geq 6$/group; (E) *Perforin-1* gene expression in TILs sorted from treated animals ($n = 3$/group). Shown is the normalized fold expression (ΔCT) in relation to HPRT expression; error bars are the SEM of replicate wells. Individual samples were pooled before sorting, which precluded statistical assessment. VM/Dk or B6 WT mice were injected i.c. with SMA-560 cells ($n \geq 7$/group; F) and B16-F10 melanoma cells ($n \geq 10$/group; G), respectively. Arrows show initiation of treatment. Osmotic minipumps delivered IL-12Fc or PBS i.t. as indicated by a horizontal line above graph. In addition, animals received i.p. injections with αCTLA-4 mAbs or PBS, indicated by vertical tick marks; two independent experiments pooled. Survival statistics according to Log-rank test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (F) p(PBS/PBS vs. IL-12Fc/αCTLA-4) = 0.0182, p(IL-12Fc/PBS vs. IL-12Fc/αCTLA-4) = 0.0155, p(PBS/αCTLA-4 vs. IL-12Fc/αCTLA-4) = 0.0177. (G) p(PBS/PBS vs. IL-12Fc/αCTLA-4) = 0.0174. Histology below survival curves shows tumor burden at initiation of treatment, overview pictures. and higher magnification pictures of the indicated regions (arrowheads). Bars: 100 µm (F); 200 µm (G).

To understand the mechanism by which IL-12Fc/αCTLA-4 combination therapy alters the composition of TILs, most notably the drop in T reg cells and increase in T eff cells, we analyzed proliferation of TILs upon treatment. By assessing BrdU incorporation and Ki67 labeling, we found that IL-12Fc, as well as IL-12Fc/αCTLA-4 combination therapy led to a selective drop of $BrdU^+$ T reg cells during the second week of treatment. At day 35, the T eff cell population did however display a higher percentage of Ki67-positive cells (Fig. 4 C), resulting in a significant increase in the ratio of $FoxP3^-$ versus $FoxP3^+$ CD4 T cells regarding BrdU incorporation and Ki67 labeling (Fig. 4 D). We did not observe significant differences in Annexin V labeling, a marker for apoptosis, most likely due to the fact that apoptotic cells are rapidly cleared by phagocytes in vivo (unpublished data). The mechanism by which CTLA-4 blockade inhibits T reg cell function remains a subject of intense debate (Walker and Sansom, 2011). Recent studies demonstrate that selective opsonization of T reg cells with CTLA-4 antibodies can elicit potent antibody-dependent cell-mediated cytotoxicity (ADCC) within the tumor site (Selby et al., 2013; Simpson et al., 2013). In regard to the tumor-suppressing effector cells, IL-12 increased the expression of perforin-1 in CD8 T cells and NK cells, but not in CD4 cells (Fig. 4 E), indicating that CTLs are ultimately responsible for tumor control.

We next tested the treatment regimen in a different model of GB. SMA-560 is derived from a spontaneous murine astrocytoma (Uhl et al., 2004). Here, we initiated treatment on day 7 after tumor cell implantation. Also, in this genetically different mouse strain (VM/Dk), we found the combination therapy to confer a significant survival advantage (Fig. 4 F).

The poor activity of CTLA-4 monotherapy observed in our study contrasts with previous studies using similar models (Fecci et al., 2007; Grauer et al., 2007; Agarwalla et al., 2012). In those studies CTLA-4 blockade was initiated at much earlier time points after seeding, the disease course progressed at a slower pace, and/or a different mAb was used in treatments. We used a different anti–CTLA-4 mAb (9D9, IgG2b), which has been reported to confer weaker ADCC of T reg cells than the 9H10 hamster mAb used by Fecci et al. (2007; Selby et al., 2013; Simpson et al., 2013). It is therefore tempting to speculate that the combination of i.t. IL-12Fc in combination with systemic CTLA-4 blockade through a strong ADCC-inducing mAb may yield even better synergy. To expand our treatment strategy toward other types of cancers, we explored intracranial implantation of B16:F10 melanoma cells. B16 is an extremely aggressive melanoma cell line and poorly immunogenic (Becker et al., 2010). In this rapidly progressing model of intracranial melanoma, only combination therapy led to a significant, albeit very modest survival advantage when compared with vehicle controls (Fig. 4 G).

Systemic anti–CTLA-4 treatment is FDA-approved for metastatic melanoma based on clinical trials demonstrating clinical benefit (Hodi et al., 2010) and has been further tested for various other solid cancers (Grosso and Jure-Kunkel, 2013). In light of the data presented here, a combination therapy consisting of systemic checkpoint blockade and local administration of IL-12 is a highly promising candidate for swift clinical translation in GB.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from Janvier; *b2m*$^{-/-}$, *Ia(b)*$^{-/-}$, *Il12rb2*$^{-/-}$, *Rag1*$^{-/-}$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, *Prf1*$^{-/-}$, and *Ifng*$^{-/-}$ mice were obtained from The Jackson Laboratory. *Il15ra*$^{-/-}$ mice were provided by S. Bulfone-Paus (Forschungszentrum Borstel, Borstel, Germany) and VM/Dk mice are bred in our laboratory. All animals were kept in house according to institutional guidelines under specific pathogen–free conditions at a 12-h light/dark cycle with food and water provided ad libitum. All animal experiments were performed according to institutional guidelines and approved by the Swiss cantonary veterinary office (licenses 16/2009; 65/2012).

Murine tumor cell lines. GL-261 cells (provided by A. Fontana, Experimental Immunology, University of Zurich, Zurich, Switzerland), which are syngeneic in C57BL/6 mice, were stably transfected with pGl3-ctrl and pGK-Puro (Promega) and selected with puromycin (Sigma-Aldrich) to generate luciferase-stable GL-261 cells. A single clone was isolated by limiting dilution and passaged in vivo by intracranial tumor inoculation. Subsequently, cells were transfected with pCEP4-mIgG3, pCEP4-mIl-12mIgG3, or pCEP4-mIl-23mIgG3, and cytokine production was detected by ELISA and RT-PCR, as previously described (Eisenring et al., 2010). SMA-560 spontaneous murine astrocytoma cells were characterized previously (Uhl et al., 2004). B16-F10 C57BL/6 murine melanoma cells were purchased from American Type Culture Collection.

Proliferation assay. 5,000 cells/well were plated into a 96-well plate in triplicates. Medium containing 0.5 mCi/ml of [^{3}H]thymidine was added, and 4 d later the incorporation was assessed using a Filtermate Collector (Applied Biosystems) and a scintillation counter (MicroBeta Trilux 1450; Wallac).

Expression and purification of IL-12Fc. IL-12Fc was expressed in 293T cells. The protein was purified from supernatant over a protein G column (1 ml; HiTrap; GE Healthcare) and eluted with 0.1 M glycine, pH 2.0, using a purifier (ÄktaPrime) and dialyzed overnight in PBS, pH 7.4. Concentration and purity of IL-12Fc were measured by ELISA (OptEIA mouse IL-12/23p40; BD) and SDS-PAGE (silver staining and immunoblotting). IL-12Fc was detected with a rat anti–mouse IL-12p40 antibody (C17.8; BioExpress) and a goat anti–rat HRP coupled antibody (Jackson ImmunoResearch Laboratories).

Functional characterization of IL-12Fc. Splenocytes isolated from WT or *Il12rb2*$^{-/-}$ animals were plated in duplicates at a density of 10^5 cells/well in RPMI medium (GIBCO BRL Invitrogen) supplemented with 10% fetal calf serum in 96-well plates and stimulated with either recombinant murine IL-12 (PeproTech) or IL-12Fc. Splenocytes were cultured in the presence of 0.5 µg/ml of anti-mouse CD3 (2C11, BioExpress) and anti-CD28 antibodies (37N, BioExpress). After two days of culture supernatant was harvested and IFN-γ detected with an anti-mouse IFN-γ ELISA kit (OptEIA mouse IFN-γ BD).

Orthotopic glioma inoculation. In brief, 6–10-wk-old mice were i.p. injected with fluniximin (Biokema; 5 mg/kg body weight) before being anesthetized with 3–5% Isoflurane (Minrad) in an induction chamber. Anesthesia on the stereotactic frame (David Kopf Instruments) was maintained at 3% Isoflurane delivered through a nose adaptor, A blunt-ended syringe (Hamilton; 75N, 26s/2"/2, 5 µl) was placed 1.5 mm lateral and 1 mm frontal of bregma. The needle was lowered into the burr hole to a depth of 4 mm below the dura surface and retracted 1 mm to form a small reservoir. Using a microinjection pump (UMP-3; World precision Instruments Inc.), 2×10^4 GL-261 (500 SMA-560 or 50 B16-F10) cells were injected in a volume of 2 µl at 1 µl/min. After leaving the needle in place for 2 min, it was retracted at 1 mm/min. The burr hole was closed with bone wax (Aesculap; Braun) and the scalp wound was sealed with tissue glue (Indermil; Henkel).

In vivo bioluminescent imaging. Tumor-bearing mice were injected with D-Luciferin (150 mg/kg body weight; Caliper Life Sciences). Animals were transferred to the dark chamber of a Xenogen IVIS 100 (Caliper Life Sciences) imaging system and luminescence was recorded. Data were subsequently analyzed using Living Image 2.5 software (Caliper Life Sciences). A circular region of interest (ROI; 1.46 cm diam) was defined around the tumor site and photon flux of this region was read out and plotted.

Treatment of established gliomas. At d 21 after implantation of the GL-261luc:Fc glioma cells, the tumor-bearing animals were evenly distributed among experimental groups based on their ROI-photon flux. Animals with an ROI flux of less than 10^5 p/s were considered as non- or slow-takers and excluded. Osmotic pumps (model 2004, 0.25 µl/h; Alzet) were filled with murine IL-12Fc (8.33 ng/µl in PBS, 50 ng/24 h) or PBS alone and primed at 37°C in PBS. Implantation of osmotic minipumps has been described previously (vom Berg et al., 2012). The previous burr hole of the glioma injection was located, the bone wax and periosteal bone was removed, and the infusion cannula was lowered through the burr hole 3 mm into the putative center of the tumor. Pumps were explanted at day 49. Five doses of anti–mouse-CTLA-4 mouse-IgG2b antibodies (clone 9D9; BioExpress) or an equivalent volume of PBS were i.p. injected at days 1 (200 µg), 5 (100 µg), 8 (100 µg), 14 (100 µg), and 21 (100 µg) after pump implantation. For treatment of established B16-F10–derived brain tumors, pumps were implanted at day 5 after injection, for treatment of established SMA-560 tumors, pumps (model 1004, 0.25 µl/h; Alzet, filled with 41.65 ng/µl IL-12Fc in PBS; 250 ng/24 h) were implanted at day 7 after injection and explanted at day 21. Anti–mouse-CTLA-4 mouse-IgG2b antibodies (clone 9D9; BioExpress) were administered according to the aforementioned dosing scheme.

Survival analysis. Tumor-bearing animals were monitored by BLI, checked for neurological symptoms and weighed weekly until day 21 after glioma inoculation. From day 21 onwards animals were checked daily. Animals that showed symptoms such as apathy, severe hunchback posture, or weight loss exceeding 20% were euthanized. SMA-560 or B16-F10 tumor-bearing mice were scored daily starting at day 14 (or day 5, respectively) until the end of the experiment according to the same scheme.

Flow cytometry. The following antibodies were used for flow cytometric analyses: anti-CD45 (30-F11; BioLegend), anti-CD11b (M1/70; BD), anti-CD4 (Gk1.5; BioLegend or BD), anti-CD8 (53–6.7; BioLegend or BD), anti-CD3 (17A2; BioLegend), anti-H-2D(b) (24–14–8; eBioscience), anti–I-A(b) (AF6-120.1; BD), anti-NK1.1 (PK136; BD), anti-FoxP3 (FJK-16s; eBioscience), anti-BrdU (Bu20a; eBioscience), anti-Ki67 (SOLA15; eBioscience), Annexin V (eBioscience), anti-CTLA-4 (UC10-F10; BD), and anti–IFN-γ (XMG1.2; BD). For the exclusion of dead cells, we used the Zombie Aqua fixable viability kit (BioLegend). Doublets were excluded based on FSC-A/FSC-H. The frontal part of the tumor-bearing cerebral hemisphere was harvested, and cells were prepared for flow cytometry as described previously (vom Berg et al., 2012). Cells were restimulated for 3 h at 37°C and 5% CO_2 in RPMI 1640 medium. RPMI 1640 was supplemented with 10% FCS and phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (500 ng/µl), and brefeldin A (1 µl/ml medium; GolgiPlug; BD). For in vivo proliferation analysis, BrdU (Sigma-Aldrich) was added to drinking water (80 mg/100 ml) and changed every second day during the second week of treatment (day 28–35). For analysis of proliferation or apoptosis, cells were not restimulated. Intracellular cytokine, transcription factor, CTLA-4, BrdU, and Ki67 staining was performed using the eBioscience FoxP3 staining buffer set following the manufacturer's instructions. Acquisition was performed on a LSRII Fortessa flow cytometer (BD), sorting was performed on a FACSAria II. Data analysis was performed using FlowJo Version 9.6.4 (Tree Star).

Complementary DNA synthesis and quantitative real-time PCR. Respective populations were FACS sorted, gating on NK cells (live singlets, $CD45^{hi}NK1.1^{+}CD3^{-}$), CD4T cells (live singlets, $CD45^{hi}NK1.1^{-}CD3^{+}CD11b^{-}CD4^{+}$), and CD8 T cells (live singlets, $CD45^{hi}NK1.1^{-}CD3^{+}CD11b^{-}CD8^{+}$). Cells were frozen immediately, lysed and RNA isolated. Poly-d(T) primers were used for synthesis of complementary DNA. TaqMan probes and primers for hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Perforin (*prf1*) were used for quantitative real-time PCR using a CFX384 Cycler (Bio-Rad Laboratories). Subsequent analyses were performed with Bio-Rad CFX Manager software using the ΔCt method. The specificity of amplification was also assessed by gel electrophoresis.

Histology. For histology, animals were euthanized with CO_2, transcardially perfused with ice-cold PBS, and decapitated. The brain was removed and the frontal part of the cerebrum was embedded in optimal cutting temperature compound (O.C.T.; Sakura) and snap-frozen in liquid nitrogen. Immunostaining of cryosections was performed as described previously (Eisenring et al., 2010). Alternatively, sections were stained with hematoxylin and eosin. Brains were also fixed in 4% formalin, embedded in paraffin and 2–3 µm sections were processed. Pictures were generated using an Olympus BX41 light microscope equipped with an Olympus ColorView IIIu camera and Olympus cell B image acquisition software. Whole slides were also scanned with a Zeiss Mirax Midi slidescanner, equipped with a 20× objective (NA 0.8) and 3CCD color camera (1360 × 1024 pixel, size 0.23 µm) and analyzed using Panoramic viewer 1.14.50 RTM and the HistoQuant plugin (both 3DHISTECH). Tumor boundaries were manually outlined and either area covered by staining and/or number of objects stained where quantified using the batch processing function. Images shown in figures were processed with Adobe Photoshop CS5.

Statistical analysis. For all nonsurvival analyses of two experimental groups, an unpaired, two-tailed Student's *t* test was performed. For all nonsurvival analyses of three or more experimental groups, a one-way ANOVA with Bonferroni posttest was performed. For statistical analysis of Kaplan-Meier survival curves, a Log-rank (Mantel-Cox) test was used to calculate the p-values indicated in respective experiments. P-values <0.05 were considered statistically significant and indicated in figures as asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All quantitative analysis was performed with GraphPad Prism version 5.0a for Mac OSX (GraphPad Software, Inc).

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B. Becher, J. vom Berg, and The University of Zurich hold a patent application entitled "Combination medicament comprising IL-12 and an agent for blockade of T cell inhibitory molecules for tumor therapy" (PCT/EP2012/070088). The authors have no additional financial interests.

Author contributions: B. Becher and J. vom Berg conceived the study and co-wrote the manuscript. B. Becher designed and J. vom Berg designed and performed experiments. M. Vrohlings, S. Haller, A. Haimovici, P. Kulig, and A. Sledzinska performed experiments. M. Weller provided animals and cells and helped with data interpretation. All the authors read and approved the manuscript.

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15. Declaration

I herewith declare that this thesis was written by myself, using the given references only. The experiments were performed by myself with the help of Sonia Tugues, Claudia Haftmann, Brian Leung, Anna Sassi, Jennifer Jaberg, Sabrina Nemetz, Jan Candreia and Mirjam Lutz. All people are current or former members of the Institute of Experimental Immunology.

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